UNIVERSIDADE FEDERAL DO PARANÁ

FABIANA TONIAL

BIOPROSPECÇÃO DE METABÓLITOS SECUNDÁRIOS DE ENDÓFITOS DE *Schinus terebinthifolius*

CURITIBA
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Tese apresentada ao Programa de Pós-Graduação em Microbiologia, Parasitologia e Patologia da Universidade Federal do Paraná, como requisito parcial para obtenção do grau de doutor em Ciências Biológicas.

Profª Drª Chirlei Glienke

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TERMO DE APROVAÇÃO

"BIOPROSPECÇÃO DE METABÓLITOS SECUNDÁRIOS DE ENDÓFITOS DE Schinus Terebinthifolius"

Por

FABIANA TONIAL

Tese aprovada como requisito parcial para obtenção do grau de Doutor no Curso de Pós-Graduação em Microbiologia, Parasitologia e Patologia, pela Comissão formada pelos professores:

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Curitiba, 29 de setembro de 2014.

Ao meu pai, Jenoino Tonial, que foi meu grande incentivador e nunca deixou de lutar pelos sonhos. E à minha mãe, Maria Alda M. Tonial, exemplo de coragem.

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RESUMO

A obtenção de novos compostos bioativos com diferentes mecanismos de ação, menores efeitos indesejáveis, maior segurança na utilização e maior eficácia é uma necessidade constante na saúde, agronomia e indústria. Essa busca é indiscutivelmente importante tratando-se de compostos antimicrobianos devido a capacidade dos microrganismos tornarem-se resistentes aos mesmos. A bioprospecção é uma das ferramentas utilizadas para a obtenção de produtos naturais com essa ação. Uma das fontes promissoras utilizadas nesse tipo de investigação são os metabólitos secundários produzidos por microrganismos endofíticos obtidos a partir de plantas medicinais. Deste modo, o trabalho a seguir teve como propósito a obtenção de produtos bioativos produzidos por fungos endofíticos das folhas de *Schinus terebinthifolius*, árvore popularmente conhecida como aroeira. O trabalho de Bioprospecção de Metabólitos Secundários de Endófitos de *S. terebinthifolius* foi dividido em dois capítulos voltados à otimização da produção de extratos ativos. O primeiro objetiva a obtenção de compostos contra patógenos humanos e o segundo contra o fitopatógeno *Phyllosticta citricarpa*. O primeiro capítulo, intitulado **"Antimicrobial potential of endophytic fungi from *Schinus terebinthifolius*",** explorou a capacidade de produção de extratos de interesse a partir de três fungos pertencentes aos gêneros *Alternaria, Bjerkandera* e *Xylaria,* sob diferentes condições de cultivo, comprovando a influência das fontes de carbono e nitrogênio, pH e temperatura sobre o metabolismo secundário dos microrganismos. A obtenção do extrato do fungo *Alternaria* sp. Sect. Alternata teve a escala ampliada e o mesmo foi avaliado frente à bactéria *Staphylococcus aureus* resistente à meticilina (MRSA). Duas porções do extrato bruto foram ativas contra essa bactéria. Hexadecanoato de metila e um alcaloide pirrolopirazina foram identificados nas frações ativas do isolado de *Alternaria* sp. Sect. Alternata. O segundo estudo, **"Biological activity of *Diaporthe terebinthifolii* extracts against *Phyllosticta citricarpa*"**, possibilitou a obtenção de uma fração biologicamente ativa contra o fitopatógeno. Da biodiversidade fúngica estudada dois isolados de *D. terebinthifolii* foram os mais promissores contra *P. citricarpa*. Diferentes meios de cultivo foram avaliados na seleção do isolado mais eficiente, o qual foi escolhido para a produção do extrato em maior escala. O meio sólido arroz foi o mais apropriado. Foram identificados alguns compostos de interesse produzidos pelo endófito: álcool fenetílico, verbanol, hexadecanoato de metila e acetato de verbenil. De modo geral, os fungos endofíticos isolados das folhas de aroeira apresentaram interessante potencial biotecnológico, tanto para a área médica como agronômica, pela produção de metabólitos secundários com atividade antimicrobiana.

Palavras-chave: endófitos - metabólitos secundários - *Schinus terebinthifolius* - atividade antimicrobiana

ABSTRACT

The obtainment of new bioactive compounds with different mechanisms of action, minor side effects, more effective and safest is a constant need in health, agronomy and industry. This search is unquestionably important in the case of antimicrobial compounds because of the ability of microorganisms become resistant to them. The bioprospection is one of the tools used to obtain natural products with this action. One of the promising sources used in this type of investigation are secondary metabolites produced by endophytic microorganisms obtained from medicinal plants. Thus, the following work had as purpose obtaining bioactive products produced by endophytic fungus from the leaves of *Schinus terebinthifolius,* tree popularly known as aroeira. The work "Secondary Metabolites Bioprospection of *S. terebinthifolius* Endophytes" was divided into two chapters. The first one focused in the optimization of active extracts production against human pathogens and the second in the obtainment of a product against the phytopathogen *Phyllosticta citricarpa*. The first chapter, entitled "**Antimicrobial potential of endophytic fungi from *Schinus terebinthifolius***", explored the capacity of production of interest extracts using three fungi belonging to the genera *Alternaria, Bjerkandera* and *Xylaria* under different culture conditions demonstrating the influence of carbon and nitrogen sources, pH and temperature on the secondary metabolism of these microorganisms. The scale production of *Alternaria* sp. Sect. Alternata extract was expanded and the product has been evaluated against the methicillin-resistant *Staphylococcus aureus* (MRSA). Two active portions against the microorganism were isolated from the crude extract. Methyl hexadecanoate and a pyrrolopyrazine alkaloid were identified in the active fractions of the *Alternaria* sp. Sect. Alternata isolated. The second study, "**Biological activity of *Diaporthe terebinthifolii* extracts against *Phyllosticta citricarpa***", allowed obtaining a biologically active fraction against the phytopathogen. Of the fungal biodiversity studied two isolates of *D. terebinthifolii* were the most promising against *P. citricarpa*. Different culture media were evaluated for selecting the most efficient isolate for the production of the extract on a larger scale. The rice solid medium was the most appropriate. Some compounds of interest produced by the endophyte were identified: phenylethyl alcohol, verbanol, methyl-hexadecanoate and verbenyl acetate. In general, the endophytic fungi isolated from the leaves of aroeira showed interesting biotechnological potential, both for medical and agronomic field, by the production of secondary metabolites with antimicrobial activity.

Keywords: endophytes - secondary metabolites - *Schinus terebinthifolius* - antimicrobial activity

SUMÁRIO

1 INTRODUÇÃO

A bioprospecção, investigação que visa a obtenção de novos produtos a partir de fontes naturais, em um país com expressiva biodiversidade e que abrange uma diversidade de biomas como o Brasil deve ser considerada um tesouro nacional. O incentivo para explorar nossos recursos biológicos deve estar entre as estratégias de desenvolvimento nacional, já que sua investigação resulta em avanços científico, tecnológico e industrial. A diversidade natural brasileira deve ser protegida, porém explorada pela sua nação, pois não basta possuir a potencialidade se essa não for utilizada em benefício da sociedade. A realização de pesquisas em biodiversidade envolvendo coleta, *screening* e avaliação do potencial industrial em instituições governamentais e universidades reduz os riscos e o tempo necessários em pesquisas e desenvolvimento para as indústrias farmacêuticas, aumentando o interesse destas pela utilização de compostos naturais no desenvolvimento e comercialização de novos produtos.

Um dos valiosos recursos naturais utilizados dentro do contexto de bioprospecção são os microrganismos. Desde a descoberta da penicilina por Fleming em 1929 os microrganismos são explorados quanto a sua produção de metabólitos secundários bioativos, principalmente antibióticos, anti-inflamatórios e antitumorais. Vários dos fármacos antimicrobianos utilizados na terapêutica são provenientes do metabolismo secundário de microrganismo como a estreptomicina, o clorafenicol, a polimixina, cefalosporinas, a nistatina, a eritromicina, a vancomicina, a gentamicina (SERRA, 2010). Dos microrganismos envolvidos com a produção destas substâncias destacam-se os fungos filamentosos e os actinomicetos do gênero *Streptomyces* (BÉRDY, 2005).

Uma estratégia de direcionamento da pesquisa científica alia o conhecimento popular da atividade biológica das plantas medicinais com a potencialidade de produção de metabólitos secundários de interesse por seus microrganismos endofíticos. Os endófitos são microrganismos que colonizam, em pelo menos uma etapa de seu ciclo vital, tecidos internos de plantas sem lhes causar prejuízo imediato (BACON e WHITE, 2000; AZEVEDO, 1998). Eles estão associados a descoberta de diversos novos compostos naturais de interesse para a saúde, agricultura e indústria, sendo amplamente explorados pela comunidade

científica. O exemplo clássico da comprovada relação na produção de compostos bioativos pelos endófitos presentes nas plantas é o taxol, produzido por plantas do gênero *Taxus* e também obtido de um endófito de *Taxus mairei*, o fungo *Tubercularia* sp. (WANG *et al*., 2000). Essa interação é um indício inicial de qual material biológico e potencialidade devem ter prioridade de exploração em determinada investigação.

Com o advento da biotecnologia foram iniciados trabalhos com o intuito de aprimorar a produção e extração de compostos, fazendo dos microrganismos a fonte mais produtiva para a obtenção de metabólitos ativos. As condições de cultivo são um elemento chave na descoberta de novos compostos, pois influenciam a sua produção de maneira quantitativa e qualitativa, permitindo explorar com mais profundidade o potencial dos microrganismos. Ainda, otimizar o processo de fermentação pode levar ao encontro de extratos com um menor número de compostos interferentes ou sem atividade, simplificando o processo de isolamento das estruturas de interesse, aumentando o seu rendimento e, consequentemente, reduzindo os custos para a obtenção do produto final (ZHANG e DEMAIN, 2005).

Sendo cientificamente comprovado que a aroeira (*Schinus terebinthifolius*) produz compostos antimicrobianos (MARTINES *et al.,* 1996; GUERRA *et al.,* 2000; LIMA *et al.*, 2004; DEGÁSPARI *et al.,* 2005; SCHMOURLO *et al*., 2005; LIMA *et al*., 2006; FENNER *et al.*, 2006; BOROS, 2007; JOHANN *et al.,* 2007), atividade biológica que incluiu a espécie vegetal na lista de fitoterápicos receitados pelo Sistema Único de Saúde brasileiro, a investigação da produção de metabólitos secundários com a mesma potencialidade em fungos endofíticos obtidos das folhas da planta foi realizada. Esse trabalho explorou a atividade biológica proposta sob duas perspectivas, pelo interesse na área da saúde e da agronomia. Por isso, a pesquisa foi realizada em dois segmentos, o primeiro explorando a produção por fungos endofíticos de extratos ativos principalmente contra o *Staphylococcus aureus* resistente a meticilina, e, com outro direcionamento, pelo isolamento de um extrato ativo de endófito contra o fitopatógeno *Phyllosticta citricarpa*. Tendo em vista uma melhor qualidade dos extratos obtidos pelos fungos selecionados para o estudo, as condições de cultivo foram aprimoradas.

2 REVISÃO DE LITERATURA

2.1 BIOPROSPECÇÃO

A bioprospecção é a exploração e investigação de plantas, animais e microrganismos a fim de identificar princípios ativos e ou enzimas úteis em diferentes áreas como na indústria farmacêutica e alimentícia, na agronomia entre outras (STROBEL e DAISY, 2003; TRIGUEIRO, 2002). Ela representa uma fonte inesgotável de obtenção de novos produtos naturais se considerarmos que os organismos vivos estão em constante evolução. Os produtos naturais representam 60% dos compostos classificados como *new chemical entities* (*NCEs*) ativos contra o câncer e 75% dos ativos contra doenças infecciosas obtidos entre 1981 e 2002 (DEMAIN, 2014). Demain (2014) ainda cita que das novas drogas introduzidas no mercado entre 1985 e 2005 aproximadamente metade era relacionada a produtos naturais. O Brasil e a Espanha destacam-se entre os países íbero-americanos que mais produzem conhecimento científico em bioprospecção de sua biodiversidade (LIMA e VELHO, 2008). Apesar disso, Marinho *et al*. (2008) ressaltam que uma maior articulação entre o Estado e a iniciativa privada em pesquisas de bioprospecção valorizaria a imensa biodiversidade brasileira e estimularia a indústria nacional, tendo como exemplo os incentivos em pesquisa e desenvolvimento nessa área disponibilizados pelo governo dos Estados Unidos, onde foram criadas leis de transferência de tecnologia pública para o setor privado e 90% das empresas *life sciences* executam atividades em cooperação com universidades.

Os microrganismos lideram a produção de compostos naturais úteis (DEMAIN, 2014). Uma diversidade de classes de compostos é produzida por microrganismos endofíticos, tornando-os importante segmento investigativo para a bioprospecção. Inseridos nesse contexto, os fungos endofíticos apresentam interessante produtividade, capazes de produzir grande parte dos grupos de compostos, inclusive diversas classes de substâncias antimicrobianas (MOUZA e RAIZADA, 2013).

2.2 MICRORGANISMOS ENDOFÍTICOS

As plantas apresentam microrganismos, geralmente fungos e bactérias (incluindo actinomicetos), epifíticos e endofíticos. Os microrganismos epifíticos são aqueles encontrados na superfície de tecidos e órgãos vegetais. Os microrganismos endofíticos colonizam, em pelo menos uma etapa de seu ciclo vital, tecidos internos de plantas sem lhes causar prejuízo imediato (BACON e WHITE, 2000; AZEVEDO, 1998). A distinção entre estes microrganismos, incluindo fitopatógenos (provocam danos ao hospedeiro), é puramente didática, pois seu estado depende do estágio de desenvolvimento e de condições ambientais e da planta. Fungos micorrízicos e bactérias fixadoras de nitrogênio intimamente relacionadas com plantas também são considerados microrganismos endofíticos (AZEVEDO, 1998).

Os endofíticos podem ser transmitidos de uma planta a outra horizontalmente, através de esporos, ou verticalmente, por sementes (SAIKKONEN *et al.,* 1998). Horizontalmente, os microrganismos encontram seus hospedeiros por quimiotaxia, eletrotaxia ou acidentalmente (YOU *et al.,* 1995). Com exceção das bactérias que são transmitidas pela semente, os microrganismos endofíticos penetram nos tecidos vegetais de seus hospedeiros pelos estômatos e raízes, ferimentos ou cicatrizes, ou ainda através da secreção de enzimas hidrolíticas (ESPOSITO e AZEVEDO, 2004; KOBAYASHI e PALUMBO, 2000). Após a penetração na planta os microrganismos podem permanecer próximos ao local de entrada ou disseminarem-se dentro da planta, nos espaços intracelulares ou no sistema vascular (BELL *et al*., 1995).

A população de endofíticos de uma determinada planta pode variar com o estado de saúde da planta sugerindo uma provável ação protetora de alguns destes microrganismos (REITER *et al*., 2002; YANG *et al*., 2001). Este potencial protetor dos endófitos pode ocorrer por competição de espaço e nutrientes na planta, pela produção de compostos antimicrobianos (PLEBAN *et al.,* 1995), ou indução de resistência sistêmica (M'PIGA *et al.,* 1997). Um estudo realizado na China verificou que a sazonalidade também interfere na população de endófitos mostrando que os isolados de amostras coletadas na primavera de *Heterosmilax japonica* Kunth foram mais abundantes, podendo haver um número maior de espécies, em comparação aos obtidos no verão (GAO *et al*., 2005).

Os fungos endofíticos podem produzir substâncias que alteram fenotipicamente a planta protegendo-a (MATIELLO *et al.*, 1997). A relação dos endófitos com a produção de antibióticos está na presença de metabólitos secundários (MELO, 2005).

2.2.1 Atividade biológica de fungos endofíticos

A importância dos fungos endofíticos no contexto da bioprospecção é comprovada no fato de que a partir deles foram obtidos 42% de 23.000 compostos ativos produzidos por microrganismos, seguido pelos actinomicetos (32%) (DEMAIN, 2014). Ainda, reiterando a ideia de que os fungos devem ter destaque em pesquisas de bioprospecção, o mesmo autor expõe que, o número estimado de espécies fúngicas é 5 vezes maior do que o de plantas e 50 vezes o de espécies de bactérias.

Wiyakrutta *et al*. (2004) estudaram fungos endofíticos provenientes de plantas medicinais da Tailândia, as quais possuíam isolados ativos contra *Mycobacterium tuberculosis, Plasmodium falciparum,* vírus herpes simples tipo1, células de carcinoma epidermoide oral humano e células de câncer de mama. Li *et al*. (2005) realizaram um screening das plantas medicinais chinesas na busca de fungos endofíticos com atividade antitumoral e antifúngica, 9,2% dos isolados apresentaram atividade antitumoral e 30% exibiram atividade antifúngica. As plantas tóxicas da Amazônia, *Palicourea longiflora* e *Strychnos cogens*, também tiveram seus fungos endofíticos isolados, dos quais 19 apresentaram atividade antimicrobiana (SOUZA *et al*., 2004).

Em 2000, Wang *et al*. verificaram que o taxol (antitumoral usado clinicamente), produzido por plantas do gênero *Taxus,* também podia ser obtido de um endófito de *Taxus mairei*, o fungo *Tubercularia* sp. Uma substância citotóxica, contra células de tumor epidermoide de nasofaringe, e antifúngica, contra *Candida albicans*, foi extraída do fungo *Neoplaconema napellum*, endofítico da *Hopea hainanensis*, por Wang *et al.* (2006). Isolados fúngicos de *Quercus variabilis* mostraram atividade antifúngica e antibacteriana, sendo que a brefedina A, composto antimicrobiano, foi extraída de uma de suas linhagens endofíticas de *Cladosporium* sp. (WANG *et al*., 2007). Os mesmos autores (2008) também descreveram a atividade de metabólitos secundários de *Penicillium* sp., isolado das folhas de *Hopea hainanensis*, relatando a ação destes contra *C. albicans* e

Aspergillus niger e também contra um tumor epidermoide. Lin *et al.* (2007) isolaram os fungos endofíticos da planta *Camptotheca acuminata* e relataram que destes 27,6% possuíam atividade contra microrganismos, 4,0% eram citotóxicos e 2,3% faziam inibição da protease. Substâncias anti-*Helicobacter pylori* foram extraídas de fungos endofíticos da planta *Cynodon dactylon*, uma planta medicinal. Tais fungos também inibiram o crescimento de *Sarcina lutea, S. aureus* e *C. albicans* (LI *et al*., 2005). Em 2005, Sette *et al.* isolaram fungos endofíticos de *Coffea arabica* e *C. robusta* (planta do café) com atividade antimicrobiana contra *Salmonella choleraesuis*, *S. aureus*, *P. aeruginosa* e quatro sorotipos de *E. coli.* Um isolado de *Aspergillus niger*, endofítico da planta *Cyndon dactylon*, produziu metabólitos com atividade antitumoral e antimicrobiana (SONG *et al*., 2004). De uma planta conhecida pela sua atividade contra o *Trypanosoma*, a *Trixis vauthieri* DC (*Asteraceae*), foi isolado um fungo, *Alternaria* sp., o qual produziu um extrato que inibiu em 99% o patógeno (COTA *et al*., 2008).

Como a produção de metabólitos secundários é individualizada, a busca da bioatividade em diversos isolados de um único gênero fúngico salienta a necessidade da exploração contínua dessa fonte de novos compostos naturais, como evidenciado por Gomes-Figueiredo *et al.* (2007) no trabalho que verificou que dentre treze, apenas dois isolados de *Pestalotiopsis* spp., endofíticos de espinheira-santa (*Maytenus ilicifolia*), apresentaram atividade antibacteriana, inclusive contra a bactéria *Staphylococcus aureus* resistente à meticilina (MRSA). Em Glienke *et al.* (2012) também observa-se espectros de atividade completamente opostos entre extratos obtidos de dois fungos pertencentes ao gênero *Diaporthe* isolados de folhas de *S. terebinthifolius*.

2.2.2 Classes de compostos antimicrobianos produzidos por fungos endofíticos

Os metabólitos secundários produzidos por fungos endofíticos compreendem uma diversidade de classes químicas, dentre aquelas com atividade antimicrobiana podemos citar: compostos alifáticos, compostos fenólicos (fenóis e ácidos fenólicos, derivados da isocumarina, flavonoides e lignanas, quinonas), alcaloides (derivados do indol, aminas e amidas), peptídeos, policetídeos, esteroides, terpenoides (principalmente sesquiterpenos, diterpenos e triterpenos) (YU *et al*., 2010; MOUZA e RAIZADA, 2013). Segundo Mouza e Raizada (2013), os

terpenoides e policetídeos estão entre os compostos antimicrobianos mais frequentemente isolados, enquanto os flavonoides e as lignanas são mais raros. Yu *et al*. (2010) destacam os alcaloides como um produto comumente obtido pelo metabolismo secundário de fungos endofíticos.

2.3 METABÓLITOS SECUNDÁRIOS

O metabolismo primário, tanto de plantas como de microrganismos, é aquele que garante as substâncias (ácidos graxos, proteínas, carboidratos) responsáveis pelo crescimento e manutenção das células. Já, o metabolismo secundário pode ser visto como a produção de compostos com funções específicas para o organismo, como para a reprodução, para a proteção contra outros microrganismos, contra radiação, entre outras, nem todas elucidadas. Dependendo da necessidade do composto secundário, a habilidade em produzí-lo pode ser perdida ou cessada temporariamente. Calvo *et al.* (2002) e Okafor (2007) descrevem algumas hipóteses que tentam justificar a produção dessas substâncias:

- de que as substâncias são produtos das vias do metabolismo primário e da quebra das macromoléculas e que serviriam como um estoque de material nutritivo;

- que inibiriam a competição de outros microrganismos por nutrientes e impediriam infecções;

- de que, ao serem produzidos no esgotamento de um nutriente vital, manteriam os mecanismos essenciais para a multiplicação celular ativando outras vias para a sua produção;

- de que, no caso de uma super-produção de algum metabólito primário, o excesso seria convertido no metabólito secundário e excretado da célula evitando a morte celular, atuando como um mecanismo de detoxificação;

- de que as substâncias regulariam algumas mudanças morfológicas, principalmente para os microrganismos, as quais estão comumente associadas à esporulação, ativando-a ou produzindo pigmentos para as suas estruturas;

- de que elas são formadas dentro de uma série de reações bioquímicas que ocorrem nas células, e que quando o resultado é um produto que favoreça a sobrevivência este mecanismo é incorporado às reações primárias, como os pigmentos, que protegem contra os danos da luz ultravioleta e são importantes fatores de virulência.

Esse tipo de metabolismo ocorre teoricamente após a fase de crescimento celular, quando há exaustão de um nutriente, biossíntese ou adição de um indutor, e/ou pelo decréscimo da taxa de crescimento. Ele é característico de algumas espécies de plantas e microrganismos (fungos, líquens e actinomicetos são os microrganismos com maior capacidade de produzi-los) e é regulado por determinados nutrientes, taxa de crescimento, *feedback*, indução e inativação enzimática, porém ainda há pouco conhecimento de suas vias em relação ao que se sabe sobre metabolismo primário (OKAFOR, 2007). A produção de um determinado metabólito, incluindo os secundários, principalmente em fungos e plantas, é tão limitada a uma espécie que há estudos que buscam traçar um perfil químico dos metabólitos das mesmas em busca da classificação e identificação do isolado ou da planta. Tais estudos se enquadram em um dos conceitos de quimiotaxonomia (FRISVAD *et al.*, 2008).

Os metabólitos secundários passaram a ser explorados pela sua importância nas áreas da saúde e da economia, já que se comportam como substâncias antibióticas, pigmentos, toxinas, feromônios, enzimas inibidoras, agentes imunomoduladores, agonistas, antagonistas, pesticidas, antitumorais, promotores de crescimento de planta e animais (OKAFOR, 2007). Porém, nem todos são benéficos aos seres humanos. Um exemplo de um produto natural deletério são as micotoxinas (CALVO *et al.,* 2002). A maior fonte de metabólitos secundários bioativos são os fungos, que se destacam ecologicamente pelas suas interações químicas. Entre eles os mais estudados quimicamente estão: *Penicillium expansum* (patulina, citrinina, chaetoglobosinas, roquefortine, expansolides, communesinas, geosmina, e fumaryl-D,L-alanina - ANDERSEN *et al.*[1], 2004; FRISVAD *et al*.[2], 2004, *apud* FRISVAD *et al.*, 2008), *Fusarium poae* (trichothecenes, culmorinas, aurofusarina, fusarinas, beauvericina, e enniatinas - THRANE *et al*.[3], 2004, *apud* FRISVAD *et al.*, 2008) e *Alternaria gaisen* (ácido tenuazonico, tentoxina, altertoxina A, alternarióis, toxina AK, e altersetina – ANDERSEN *et al*.[4], 2005, *apud* FRISVAD *et al.*, 2008).

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2.4 FERMENTAÇÃO

Fermentação, no sentido industrial da palavra, é o cultivo de um microrganismo que não permite contaminação e fornece as condições necessárias para a produção máxima do metabólito desejado, podendo ou não ser secundário (OKAFOR, 2007). De acordo com o mesmo autor, as fermentações podem ocorrer em meios líquidos ou sólidos. No meio sólido pode haver a produção de compostos diferentes do líquido por permitir a produção de compostos relacionados com o processo de esporulação (CALVO *et al*., 2002).

Como os microrganismos isolados da natureza geralmente produzem níveis extremamente baixos de metabólitos secundários, a produção destes compostos em um rendimento satisfatório deve ser induzida em laboratório.

Para isso, os processos metabólicos são controlados por fontes de carbono, nitrogênio, fósforo, metais, indutores, regulação por *feedback*, taxa de crescimento e adição de enzimas (DEMAIN, 2006). O controle da temperatura e do pH, na qual há uma produtividade ótima do metabólito, também são fatores importantes que devem ser estabelecidos para a fermentação. A variação na produção dos compostos depende tanto dos fatores ambientais quanto da genética do microrganismo (MARTIN e DEMAIN, 1980; OKAFOR, 2007).

Tendo como objetivo a produção de compostos bioativos, é importante lembrar que nem sempre as melhores condições de crescimento de um microrganismo são as mais favoráveis à produção de metabólitos com atividade, como os exemplos do uso de glicose, ótima fonte de carbono para o desenvolvimento de microrganismos, porém redutora de uma série de compostos de interesse (actinomicina, cefalosporinas, alcaloides do ergot); e do uso de sais de amônio como fontes de nitrogênio favorável ao crescimento, mas que afeta as vias do metabolismo secundário (ZHANG e DEMAIN, 2005). O mesmo autor ressalta ainda mais o estudo das condições de fermentação afirmando que vários grupos de genes só são expressos em determinadas condições de cultivo. Dentre os processos de fermentação utilizados industrialmente estão: o processo descontínuo, o processo descontínuo alimentado e o processo contínuo. Desses destacamos o processo descontínuo, que é o mais tradicional, por apresentar menor risco de contaminação, facilidade de operação e concentração elevada de produto no meio ao fim da fermentação (SCHMIDELL *et al*., 2001).

2.5 *Schinus terebinthifolius* Raddi

É um exemplar da família Anacardiaceae que apresenta as seguintes sinonímias botânicas: *Schinus aroeira* Vell, *Sarcotheca bahiensis* Turcz., *S. antiarthritica* Mart., *S. mucromulata* Mart., *S. chichita* Speg., *S. lentiscifolia* e *S. rhoifolus* Mart. (LORENZI, 2002). Popularmente esta árvore é conhecida como aroeira, aroeira pimenteira, aroeira precoce, aroeira do campo, aroeira da praia, aroeira negra, aroeira branca, aroeira vermelha, aroeira mansa, aroeira do brejo, aroeira do sertão, fruto de raposa, fruto de sabi, coração de bugre, cambuí, bálsamo, aroeira do campo, aroeira de sabiá, aroeira do Paraná, aguaraiba e careiba (LORENZI, 2002; STASI e LIMA, 2002; LIMA *et al*., 2004; RIBAS *et al*., 2006).

Schinus terebinthifolius é uma árvore que atinge de 5-12m de altura com um tronco tortuoso, revestido por uma casca grossa, de 30-60cm de diâmetro, de onde saem ramos principais repletos de ramos secundários. Sua copa é bonita e arredondada (LORENZI, 2002). Nos ramos secundários há folhas compostas imparipinadas, fortemente aromáticas, com 3-10 pares de folíolos glabros. De setembro a janeiro apresenta inflorescências paniculadas terminais, com flores pequenas e esbranquiçadas. As flores da aroeira são diclinas, dependem basicamente de insetos para o transporte do pólen, e o comprimento e a largura da inflorescência determinam o dimorfismo sexual da planta (LENZI e ORTH, 2004). Os frutos aparecem predominantemente de janeiro a julho, possuem características de drupas globosas, vermelhas e brilhantes quando maduros (FIGURA 1) (LORENZI, 2002; STASI e LIMA 2002).

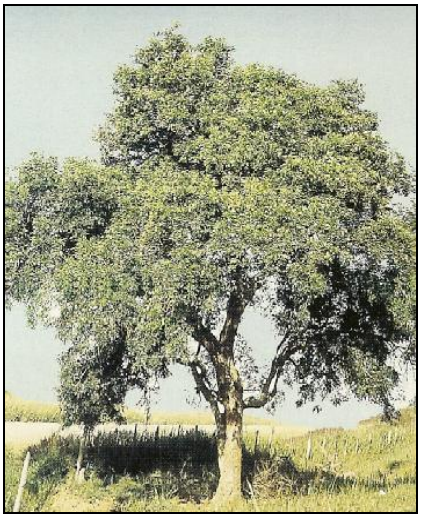


FIGURA 1 – ÁRVORE DE *Schinus terebinthifolius* Raddi
FONTE: Lorenzi (2002).

A semente de *S. terebinthifolius,* única por fruto, é reniforme, com envoltório membranáceo, liso, de coloração amarelo-clara e com uma mancha marrom escura. Ela possui reservas de proteínas e de lipídeos e apresenta um envoltório com dupla origem, um parcialmente paquicalazal (se manifesta externamente como uma mancha marrom escura) e o outro tegumentar (CARMELLO-GUERREIRO e PAOLI, 1999).

É uma planta originária da América do Sul, especialmente do sul do Brasil, do Paraguai e da Argentina. Devido seu pioneirismo e agressividade ocorre em diversas formações vegetais, incluindo terrenos secos e pobres, apesar de se adaptar melhor a beira de rios, córregos e várzeas úmidas (CARMELLO-GUERREIRO e PAOLI, 1999; LORENZI, 2002; MORGAN e OVERHOLT, 2005). Esta característica permitiu sua distribuição em todo o território brasileiro e sua presença em variados países como Peru, Uruguai, Chile, Bolívia e nos continentes europeu e asiático (RIBAS *et al*., 2006). Foi introduzida na Europa para a arborização de ruas pelo seu porte e beleza. Na França os seus frutos são utilizados na culinária, sendo conhecidos como *poivre rose*, um tipo de pimenta doce; seu cheiro é resinoso e o sabor é adstringente (devido ao tanino) e balsâmico (SCRIVANTI *et al.*, 2003; CERUKS *et al*., 2007; PLANTAS QUE CURAM, 2008).

Relata-se o uso de folhas, frutos e casca de *S. terebinthifolius* Raddi na terapêutica popular (DEGÁSPARI *et al.*, 2005). As folhas são usadas como cicatrizante de pele ou mucosa, analgésico e antipruriginoso através do uso externo de seu macerado. A administração interna da infusão das suas folhas é indicada para o reumatismo. Mastigar as folhas frescas da aroeira auxilia no tratamento de gengivites e na cicatrização da mucosa. As cascas e folhas secas da aroeira são utilizadas contra febres, diarréias, úlcera gastroduodenal, blenorragia, tosse e bronquite, problemas menstruais com excesso de sangramento, gripes, inflamações em geral e problemas do trato geniturinário, incluindo doenças venéreas, cistites e uretrites. Banhos das cascas de aroeira são indicados para o alívio da gota, reumatismo e dor ciática (MARTINES *et al.*, 1996; PLANTAS QUE CURAM, 2008).

O extrato etanólico, preparado a partir da entrecasca, é utilizado como cicatrizante de feridas cutâneas (COUTINHO *et al.,* 2006). Da casca extrai-se óleo empregado contra tumores e doenças da córnea (DEGÁSPARI *et al.*, 2005). Compressas intravaginais com o extrato aquoso desta planta são usadas para tratar cervicite e cervicovaginites (MARTINES *et al.*, 1996). Sua resina é indicada para o

tratamento de reumatismo e ínguas, além de servir como cicatrizante, analgésico, purgativo e combater doenças respiratórias (bronquite, tosse). A resina amarelo-clara proveniente das lesões das cascas é amplamente utilizada, entre os sertanejos, como tônico.

No Brasil, a resina, casca, folhas e frutos são utilizados para o tratamento de tumores. A aroeira foi utilizada pelos jesuítas que, com sua resina, preparavam o "Bálsamo das Missões", famoso no Brasil e no exterior. Um preparado dos frutos de *S. terebinthifolius* Raddi é utilizado para azia e gastrite (PLANTAS QUE CURAM, 2008). O suco do macerado de raízes é utilizado para o tratamento de tumores ganglionares (BARBOSA *et al.*, 2007). A planta inteira é utilizada, externamente, como anti-séptico no caso de fraturas e feridas expostas. Também são relatadas ações adstringente, antioxidante (fruto) e antimicrobiana (SANTOS *et al*., 2007). Em homeopatia, a planta é utilizada para o tratamento da debilidade, intumescimento linfático, inércia sexual e moléstias de pele (STASI e LIMA, 2002).

É importante ressaltar que todas as propriedades anteriormente descritas provêm da cultura popular, sendo que algumas delas já foram comprovadas cientificamente, porém outras não. Além disso, apesar dos efeitos benéficos a aroeira apresenta vários efeitos tóxicos, especialmente sob uso prolongado, o que exige cautela em sua utilização como fitoterápico. Entre os efeitos tóxicos destacam-se a dermatite alérgica em pessoas sensíveis a alquilofenóis e a atividade mutagênica (citotóxica) detectada em extratos provenientes das cascas do caule (STASI e LIMA, 2002; BARBOSA *et al.*, 2007; PLANTAS QUE CURAM, 2008).

Diversos trabalhos já confirmaram a atividade antimicrobiana de extratos obtidos a partir de *S. terebinthifolius* Raddi. Degáspari *et al.* (2005) analisaram a atividade antimicrobiana de extratos, aquoso e alcoólico, obtidos de frutos de *S. terebinthifolius* e a relacionaram com compostos fenólicos presentes nestas amostras. Os autores verificaram que o extrato alcoólico apresentou efeito inibitório sobre o crescimento de *Staphylococcus aureus* ATCC 6538 e de *Bacillus cereus* ATCC 11778, apresentando quantidade significativa da flavona apigenina, além de ácido elágico. Porém, o extrato aquoso não apresentou efeito inibitório sobre o crescimento dos microrganismos testados, sendo que nesse extrato foi detectada a presença, em pequena quantidade, da flavanona naringina. Ao contrário deste estudo, Lima *et al.* (2004) verificaram a atividade do extrato aquoso de *S. terebinthifolius* contra *S. aureus, S. epidermidis, B. cereus, Pseudomonas*

aeruginosa, Trichophyton rubrum, Microsporum canis, Epidermophyton floccosum e *Candida albicans*. Os autores discutem que esta atividade antibacteriana e antifúngica está, possivelmente, associada à presença de certos compostos químicos, em especial de taninos, além de compostos presentes em menor quantidade, como os alcaloides, esteroides, chalconas e urundeuvinas. Outros autores destacam a falta de atividade destes extratos contra *Escherichia coli* (LIMA *et al*., 2006).

Mais estudos visaram elucidar a propriedade antimicrobiana da aroeira utilizando extratos etanólicos da planta, um deles utilizando uma concentração de 30% (MARTINES *et al.,* 1996) e outro utilizando uma concentração de 80% (GUERRA *et al.,* 2000). Ambos relatam atividade inibitória contra bactérias Gram-positiva (*S. aureus*) e Gram-negativas (*E. coli, P. aeruginosa*). Martines *et al.* (1996) relatam atividade contra *B. cereus* (Gram-positivo). O fato que diferenciou estes dois estudos foi a resposta antifúngica, testada com *C. albicans*, a qual só foi efetiva com o extrato etanólico a 80%, possivelmente por permitir uma maior concentração do(s) composto(s) com atividade farmacológica no extrato.

A atividade significativa do extrato metanólico de aroeira como antiaderente da bactéria *Streptococcus mutans* em blocos de resina que simulavam a estrutura dental demonstra a capacidade de inibição da formação de biofilme do extrato da planta (BARBIERI *et al.*, 2014).

Um levantamento bibliográfico etnobotânico sobre plantas utilizadas para infecções fúngicas na medicina popular destaca o uso de *S. terebinthifolius* Raddi dentre 409 espécies de plantas (FENNER *et al.*, 2006). Outra pesquisa relacionada com a propriedade antifúngica desta planta testou seus extratos, aquoso e alcoólico, contra *C. albicans*, *T. rubrum* e *Cryptococcus neoformans*. O resultado foi de atividade contra *C. albicans* com o uso do extrato aquoso, porém quando as macromoléculas foram separadas de outros metabólitos houve perda de atividade (SCHMOURLO *et al*., 2005). Um trabalho relacionado com a atividade antimicrobiana de um extrato de *S. terebinthifolius* Raddi também verificou que o fracionamento do mesmo induzia a perda da capacidade de inibição de bactérias (BOROS, 2007). Johann *et al.* (2007) verificaram que o extrato etanólico das folhas de *S. terebinthifolius* apresentou potencial atividade antifúngica contra *Candida glabrata* e *Sporothrix schenckii*. Neste mesmo estudo a análise fitoquímica preliminar

dos extratos de *S. terebinthifolius* mostrou a presença de compostos biologicamente ativos como flavonoides, triterpenos, esteroides e taninos.

Há certas contradições no espectro de ação dos extratos de aroeira de acordo com os estudos acima descritos dependentes do modo de obtenção dos mesmos, porém a atividade antimicrobiana de extratos de *S. terebinthifolius* Raddi é inquestionável.

Schinus terebinthifolius Raddi apresenta microrganismos endofíticos, os quais podem estar relacionados com a atividade fitoterápica da planta. Um estudo já associou a variação na produção de fitoquímicos com a variação da microbiota da planta (LIMA, 2008). Ainda no estudo realizado por Lima (2008), os principais gêneros fúngicos isolados das plantas que apresentaram produção de princípios ativos foram *Phomopsis, Pestalotiopsis, Colletotrichum* e *Phyllosticta*, porém nas plantas sem produção de princípios ativos os principais gêneros endofíticos isolados foram *Phyllosticta* e *Colletotrichum*. Há pouco estudo sobre a comunidade endofítica da planta *S. terebinthifolius* Raddi. Strapasson *et al.* (2002) isolaram *Fusarium, Alternaria, Pestalotia, Penicillium, Aspergillus, Trichoderma, Chaetomium, Nigrospora, Goetrichum* e *Mucor* como endofíticos de sementes de aroeira. Lima (2008) isolou das folhas de *S. terebinthifolius* Raddi fungos do gênero *Colletotrichum*, *Phomopsis, Pestalotiopsis* e *Phyllosticta*.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Prospectar fungos endofíticos das folhas de *Schinus terebinthifolius* produtores de extratos com atividade antimicrobiana e identificar microrganismos e compostos produzidos.

3.2 OBJETIVOS ESPECÍFICOS

- Aperfeiçoar as condições de cultivo para a extração de metabólitos secundários de interesse de alguns endófitos das folhas de aroeira;
- Avaliar a atividade antimicrobiana dos extratos obtidos a partir dos endófitos;
- Identificar os compostos majoritários presentes nos extratos ativos;
- Identificar os principais microrganismos produtores de compostos de interesse ao nível de espécie.

4 POTENCIAL ANTIMICROBIANO DE FUNGOS ENDOFÍTICOS DE *Schinus terebinthifolius*

4.1 Resumo O estudo avaliou o potencial antimicrobiano de extratos obtidos a partir de 19 fungos endofíticos isolados de *Schinus terebinthifolius*, em trabalho prévio. De acordo com características morfológicas e análise molecular foram identificados os seguintes gêneros: *Alternaria, Bjerkandera, Colletotrichum, Diaporthe, Penicillium* e *Xylaria.* Dos endófitos analisados 57.89% produziram, em pelo menos um dos processos fermentativos utilizados, compostos com atividade antimicrobiana. Nove isolados forneceram extratos ativos contra *Staphylococcus aureus*, quatro contra *Candida albicans* e dois contra *Pseudomonas aeruginosa*. Três endófitos (LGMF626 - *Alternaria* sp., LGMF673 - *Xylaria* sp. and LGMF713 - *Bjerkandera* sp.) tiveram as condições de cultivo otimizadas. Foram estudadas as fontes de carbono e nitrogênio, pH inicial, temperatura e tempo de incubação. Em geral, a galactose foi a melhor fonte de carbono e a acidificação do meio proporcionou os melhores resultados de atividade contra *Candida albicans*. Um isolado de *Alternaria* sp. Sect. Alternata produziu, após fermentação em larga escala, um extrato com duas porções ativas contra *S. aureus* resistente à meticilina, uma com elevada atividade (concentração inibitória mínima 18,52 µg/mL) e outra com atividade moderada (concentração inibitória mínima 55,55 µg/mL). Nas frações ativas do isolado de *Alternaria* sp. Sect. Alternata foram detectados os compostos hexadecanoato de metila e um alcaloide pirrolopirazina. Os fungos endofíticos de *S. terebinthifolius*, uma planta medicinal brasileira, mostraram ser uma fonte promissora de compostos antimicrobianos.

Palavras chave: Endófitos - Aroeira - *Alternaria* - Metabólitos secundários - Otimização

4 ANTIMICROBIAL POTENTIAL OF ENDOPHYTIC FUNGI FROM *Schinus terebinthifolius*

4.1 Abstract In this study we analyze the antimicrobial extract production of 19 fungi isolated from *Schinus terebinthifolius* in a previous work. The genera identified were *Alternaria, Bjerkandera, Colletotrichum, Diaporthe, Penicillium* and *Xylaria* according to morphological and molecular analysis. From endophytes analyzed 57.89% produced active extracts with antimicrobial activity in at least one of the fermentation processes. Nine isolates produced active compounds against *Staphylococcus aureus*, four against *Candida albicans* and two against *Pseudomonas aeruginosa*. Three endophytes (*Alternaria* sp. Sect. Alternata - LGMF626, *Xylaria* sp. - LGMF673 and *Bjerkandera* sp. - LGMF713) had the fermentation conditions optimized. The carbon and nitrogen sources, initial pH, temperature and period of incubation were analyzed. In general, galactose was the best carbon source for active extracts production, and the acidification of the medium provided the best results in terms of activity against *Candida albicans*. In large scale fermentation the *Alternaria* sp. Sect. Alternata - LGMF626 produced an extract with two active fractions against methicillin-resitant *S. aureus*, one with high activity (minimum inhibitory concentration of 18.52 µg/mL) and other with moderate activity (minimum inhibitory concentration of 55.55 µg/mL). Methyl hexadecanoate and a pyrrolopyrazine alkaloid were identified in the active extract of the *Alternaria* sp. Sect. Alternata - LGMF626. The endophytic fungi of *S. terebinthifolius*, a Brazilian medicinal plant, showed that can be a promising source of natural antimicrobial compounds.

Keywords Endophyte - Brazilian pepper – *Alternaria* - Secondary metabolites - Optimization

4.2 INTRODUCTION

Endophytic microorganisms are those that colonize internal tissues of plants in at least one stage of their life cycle without causing damage (AZEVEDO, 1998; BACON and WHITE, 2000). The endophytes are a promising source of new natural products that can hold the emerging problems in medicine, agriculture and industry, including those related to microorganisms (YU *et al*., 2010). Between the years 2008-2009 more than 100 new natural products produced by fungal endophytes were discovered (ALY *et al*., 2011). The classes of compounds with antimicrobial activity obtained from endophytic fungi include: terpenoids and polyketides as most prevalent; and alkaloids, phenylpropanoids, aliphatic compounds, and peptides (MOUSA and RAIZADA, 2013).

The development of resistance mechanisms by microorganisms, as well as the advent of emerging pathogens, makes the search for new antimicrobial compounds a continuing need. However, in a period of 30 years, only two truly novel classes of antibiotics, oxazolidinones and cyclic lipopeptides, have entered the market (WHO, 2012). Among the resistant microorganisms stands methicillin-resistant *Staphylococcus aureus* (MRSA), the major cause of community-acquired antibiotic resistant infections. Even though the MRSA has already been described for more than 50 years, the challenges faced in obtaining and implementing new drugs still make this pathogen a health problem worldwide (MOELLERING, 2012). Because of this, the following work has prioritized the study of compounds active against MRSA. In the initial stage of the study the spectrum of the extracts analyzed was larger, comprising a Gram-positive organism (*S. aureus*), a Gram-negative (*Pseudomonas aeruginosa* - also important by the intrinsic resistance) and a yeast (*Candida albicans*), to assist the selection of endophytes that provides the best diversity in bioactive compounds. The Gram-negative pathogen chosen is a common nosocomial microorganism with intrinsically high resistance to many antimicrobials (POLLE, 2011). While the yeast is the major infectious fungal agent (KABIR *et al.,* 2012).

The Brazilian pepper (*Schinus terebinthifolius*), or "aroeira", is a medicinal plant originated from South America and is known for its antimicrobial activity (DEGÁSPARI *et al*., 2005). Medicinal plants are an important source of endophytes

with biological activities (YU *et al.*, 2010). The interest in the medicinal property of the plant mentioned above, and the exploration of endophytes biodiversity and bioactive compounds led to this paper. Knowing that the metabolites produced by fungi can either be retained in the cellular structures or be released into the culture medium as well as the culture conditions influence in its production (OKAFOR, 2007), different ways to obtain the extracts were used in an attempt to exploit the most of the bioactive compounds production potential from the selected endophytes.

In a previous work, a total of 128 endophytic fungi were taken from leaves of *Schinus terebinthifolius* in Brazil (GLIENKE *et al*., 2012). Nineteen of these fungi were selected by the macromorphological aspects and by the analysis of pairing assays against fungi and bacteria (TONIAL, 2010; GLIENKE *et al.,* 2012). These preliminary studies led us to the investigation about the production of antimicrobial secondary metabolites.

4.3 MATERIALS AND METHODS

4.3.1 ENDOPHYTES IDENTIFICATION

The nineteen endophytic fungi from leaves of *Schinus terebinthifolius* selected were identified by standard morphological techniques (fungal slide culture and macroscopy) and by direct sequencing of fungal ITS regions (ITS1-5.8S-ITS2 of rDNA gene).

4.3.1.1 Morphological characterization

Each isolated was grown over a piece of potato dextrose medium (1 cm X 1 cm) overlapped with a laminula inside a sterilized Petri dish. After 5-15 days at room temperature, the laminula was removed and visualized in optical microscopy (FISCHER and COOK, 2001). The fungal reproduction structures found were compared with a microscopic identification key. The microscopic identification key used was described by Watanabe (2002). The macromorphology of the strain (verse and reverse color, aspect, side and edge of the colony) growed in potato dextrose agar (PDA) and malt extract agar (MEA) also aided to the identification.

4.3.1.2 Molecular characterization

Genomic DNA was extracted from the fungus grown on PDA using Ultra-Clean™MicrobialDNA Kit (MOBio, USA) according to manufacturer's protocol. The amplification was performed using the primers V9G (DE HOOG and GERRITS VAN DEN ENDE, 1998) or ITS1 and ITS4 (WHITE *et al.,* 1990) or ITS5 with the following reaction mixture (50 µL): 0.2 mM of each dNTP, 1X Tris/HCl, 1.5 mM $MgCl_2$, 1.5 U Taq polymerase (Invitrogen Corp. USA), 0.06 µM each primer and 50ng of DNA. The PCR was processed in a Mastercycler Gradient (Eppendorf AG, Germany) with program: 94 °C for 2 min at the start followed by 35 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min and a final extension of 72 °C for 3 min. The amplified DNA was sequenced at MegaBACE with DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE (Amersham Biosciences Corp.). The consensus sequences were visually inspected using MEGA 6.06 with the aid of BioEdit 7.0 and compared with those of the GenBank database by MegaBlast.

The predominant gender of the selected endophytes, *Diaporthe* sp., were grouped according to the probable species by comparing the ITS sequences with those of the GenBank database by MegaBlast considering as possible species the ones with at least 99% of similarity to the sequence obtained for the isolated endophytic.

For the fungus selected to large scale fermentation (LGMF626) and for LGMF692 the phylogenetic tree was constructed. Muscle software was used to the alignment of multiple sequences retrieved from GeneBank. The matrix distance through sequence data was calculated according to the Kimura 2-parameter evolutive model with gamma distributed with invariant sites. The phylogenetic tree was then constructed with Maximum Likelihood technique using MEGA 6.06.

4.3.2 EXTRACTS PRODUCTION

For the 19 fungi, the fermentations were realized using four culture mediums: two liquids, Czapeck (30.0 g glucose, 2.0 g sodium nitrate, 2.0 g dipotassium phosphate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, 0.5 g ferrous sulfate and 1000 mL distilled water) and Medium for Eurimicina production – MPE (20.0 g soy ; 20.0 g glucose; 2.0 g calcium carbonate; 5.0 g sodium chloride and 1000 mL

distilled water), and two solids, rice (110.0 g of rice with 100 mL of distilled water were kept overnight then submitted to 120 ºC, 1 atm, 20 min twice) and corn (50.0 g of corn with 70 mL of distilled water were submitted to 120 ºC, 1 atm, 60 min). The microorganism was grown on Potato-dextrose agar (PDA; Himedia, India) plates for 5-14 days at 28 °C.

For liquid mediums, three discs (10 mm) of the growth were inoculated into Erlenmeyer flasks (250 ml) with 50 ml or 100 ml of medium and cultured at 28 °C and 120 rpm. The cultures with 50 ml were kept for 24 hours and those with 100 ml for 7 days. The mycelium was separated of culture medium by vacuum filtration. The filtrate was extracted with ethyl acetate (Vetec, Brazil) and the mycelium with methanol (Vetec, Brazil) (both twice the volume). The extracts obtained were: CDF (Czapeck; 1 day; filtrate), CWF (Czapeck; 1 week; filtrate), CDM (Czapeck; 1 day; mycelium), CWM (Czapeck; 1 week; mycelium), MDF (MPE; 1 day; filtrate), MWF (MPE; 1 week; filtrate), MDM (MPE; 1 day; mycelium), and MWM (MPE; 1 week; mycelium).

For solid mediums, seven discs (10 mm) obtained from the growth were inoculated into 500 ml flat-bottomed flask containing the medium (50.0 g of corn or 110.0 g of rice) and cultured for 7 and 30 days at 28 °C. The growth was macerated with methanol (Vetec, Brazil) (twice the volume), the solvent was kept for 3 days before filtration. The extracts obtained were: CW (corn; 1 week), CM (corn, 1 month), RW (rice; 1 week), and RM (rice; 1 month).

All extracts were dried over rotary evaporator and resuspended to 10 mg/ml in methanol (Merck, Germany).

4.3.3 ANTIMICROBIAL ACTIVITY

The antimicrobial activity evaluation of the extracts obtained from the endophytes filtrates (CDF, CWF, MDF and MWF) was performed by the bioautography methodology (CORRADO and RODRIGUES, 2004). Basically, the extracts (5 and 10 µL - 10 mg/mL) were adsorbed in Thin Layer Chromatography (Macherey-Nagel, GmbH Co. KG) plates subsequently coated with Muller-Hinton agar (MHA; Himedia, India) containing the pathogen. The plates were incubated at 35°C for 24 hours and revealed with Thiazolyl Tetrazolium Bromide (MTT; Sigma-Aldrich Co. USA).

To evaluate the activity of the extracts obtained through the maceration of the endophyte cells masses (CDM, CWM, MDM, MWM, CW, CM, RW and RM) an adaptation of the manual standardized by National Committee for Clinical Laboratory Standards (NCCLS, 2003a) was used: wells (6 mm) were made in each Petri dishes containing MHA (Himedia, India). Then the inoculum (10^8 UFC/ml for bacteria and 10^6 UFC/ml for the yeast) was homogeneously spread over the culture medium surface with the aid of a swab. Each well was filled with 50 µL of the solution to be tested. The dishes were cultured at 35 °C for 18 hours. The presence of growth inhibition halo around the well was considered a positive result. The experiment was carried out in duplicate.

The extracts had their antimicrobial activity tested against *Staphylococcus aureus* ATCC6538, *Pseudomonas aeruginosa* ATCC27853 and *Candida albicans* ATCC10231. As positive controls chloranphenicol (1 mg/ml for *S. aureus* and 10 mg/ml for *P. aeruginosa*) and nystatin (100000 UI/ml for *C. albicans*) were used. Methanol and saline solution were the negative controls.

The minimum inhibitory concentration (MIC)) of *Alternaria* sp. - LGMF626 active fractions was determined by the microdilution method (NCCLS, 2003b) against *S. aureus* ATCC6538 and against a clinical isolate of methicillin resistant *S. aureus* (MRSA). Serial dilutions of the fractions (50 µL) were mixed with 100 µL of Mueller–Hinton broth (MHB; Himedia, India) and 5 µL of inoculum suspension ($1x10^7$ UFC/mL) in microtiter plates. Final concentration of the test fractions ranged from 166.66 µg/mL to 0.001 µg/mL. The plates were incubated at 35 °C for 24 h. MICs were recorded by reading the lowest concentration that inhibited visible growth. The assay was performed in duplicate. In order to confirm the activity, after incubation, 10 µL of MTT 2.5 mg/mL (Sigma-Aldrich Co. USA) were added to each well, and the absence of reaction represented growth inhibition. The positive control was oxacillin, and the negatives were: medium + methanol (solvent of fractions) + inoculum, and medium + inoculum; the controls were done with constant volumes only to validate the technique. The medium sterility control was also done.

4.3.4 CULTURE CONDITIONS OPTIMIZATION

From nineteen endophytes that had the antimicrobial activity of the crude extracts evaluated three (*Alternaria* sp. - LGMF626, *Xylaria* sp. - LGMF673 and *Bjerkandera* sp. - LGMF713) were selected for optimization process. The selection criterias were: spectrum of action and yield of extracts, macromorphology and genera of fungi, and characteristics of culture medium.

The Czapeck medium was used in the optimization process. The carbon sources tested were: glucose, sucrose and galactose; the nitrogen sources were: soy flour, yeast extract, sodium nitrate and ammonium sulfate. The proportions of the components were always the same. The initial pH of the media was also evaluated; were tested pH 3.0, pH 5.5 and pH 8.5. The ideal incubation temperature was determined by analyzing the cultures at 25 ºC, 28 ºC and 35 ºC. The cultivation periods were 1, 7 and 14 days. All cultures were realized in static condition.

The microorganism was grown on PDA plates for 5-14 days at 28 °C. Then, three discs of 10 mm in diameter were cut from each growth and inoculated in 150 mL of medium (250 mL Erlenmeyers). The mycelium was separated of metabolic media by filtration. The filtrate was extracted with ethyl acetate (75 mL for each extract - Vetec, Brazil), the mycelium were extracted by maceration with 50 mL of methanol (Vetec, Brazil). All extracts were dried over rotary evaporator and resuspended to 10 mg/mL in methanol (Merck KGaA, Germany).

For the antimicrobial activity evaluation of the extracts the same methodologies previously described were used. The antimicrobial activity of these extracts was tested against *S. aureus* ATCC6538 and *C. albicans* ATCC10231.

The best culture conditions were determinate by the extracts spectrum and intensity of action evaluated by the following visual graduation of the revelator pigmentation: (-) no activity; (+) low activity; (++) moderate activity; (+++) high activity; (++++) very high activity.

4.3.5 LARGE SCALE FERMENTATION

The endophytic strain (*Alternaria* sp. - LGMF626) that presented the best spectrum of action and yield was selected for large scale fermentation. The conditions used for large scale fermentation in Czapeck medium were: glucose, ammonium sulfate, pH 5.5, 35 ºC, and 7 days in static condition. After this period, the culture was filtered to separate the mycelium from the medium; the mycelium was discarded. Six liters of the fermented medium were extracted with ethyl acetate (Vetec, Brazil). The resulted material was dried over rotary evaporator.

4.3.6 CHEMICAL COMPOSITION

The crude extract (0.17 g) of ethyl acetate obtained in large scale fermentation was subjected to a column chromatography, carried out on silica gel 60 (70-230 mesh, Merck KGaA, Germany), 30 cm x 1.5 cm, and eluted with: petroleum ether; petroleum ether: ethyl acetate (1:0.5; 1:1; 0.5:1); ethyl acetate; ethyl acetate: methanol (1:0.5; 1:1; 0.5:1); methanol. This procedure resulted in 27 fractions. Thin-layer chromatography, used to monitor fractions of chromatography, was performed on silica gel 60 G/UV_{254} (Macherey-Nagel, GmbH & Co. KG). The visualization of the TLC plates was achieved with a ultraviolet (UV) lamp (λ_{max} 254 and 365 nm) and anisaldehyde spray reagent (acetic acid:sulfuric acid:anisaldehyde, 50:1:0.5; Sigma-Aldrich Co., USA) followed by heating. All fractions were submited to bioautography methodology against *S. aureus* ATCC6538 and *C. albicans* ATCC10231. The active fractions had the antimicrobial activity evaluated against *S. aureus* ATCC6538 and against a clinical isolate of methicillin resistant *S. aureus* (MRSA) by microdilution method (described above).

To verify the chemical complexity of the active fractions they were eluted in High Performance Liquid Chromatography (HPLC) performed on a Flexar (Perkin Elmer Inc., USA) model, with autosampler and Photodiode Array (PDA) detector (Perkin Elmer Inc., USA). The portion VII was eluted with methanol:water (50:50) and the portion XVIII was eluted with water (100 %), both for 30 min, at 1 ml/min. The column used was a C_{18} (Agilent Technologies, USA), 250 x 4.6 mm, 5 µm. All HPLC solvents were of HPLC grade (Vetec, Brazil), and all other chemicals used were of

analytical grade (Vetec, Brazil). To resuspend the products was used methanol (Merck KGaA, Germany).

The active extract was analyzed by gas chromatography–mass spectrometry (GC-MS) (Shimadzu® GCMS-QP2010 Plus) and nuclear magnetic resonance (NMR) for the identification of major metabolites. To obtain the GC-MS spectrums the samples were solubilized in dichloromethane ultrapure (JT Baker, USA). It was used a fused silica capillar column (Rtx-5MS) of diphenyl (5%) and dimethyl polysiloxane (95%), 30 x 0.25 mm, 0.25 μm. Gas helium was used as the carrier gas at a flow rate of 1.02 ml/min, in split mode 1:20, with the injector at 250 ºC. The sample (1 ul) was injected into the heating ramp at initial temperature of 60 °C (0 ') up to 250 °C, heating 3 °C/minute. The detection mass range was: 40-350 m/z; the transfer line and the ion source temperatures were: 300 °C; and the system for electron impact ionization was: 70 eV. The peaks were compared with those in Adams (2007) and with the National Institute of Standards and Technology mass spectrum database. The following retention index of n-alkanes was used.

Retention index of n-alkanes used in gas chromatography–mass spectrometry (GC-MS):

n-alkane	Retention time (s)
9	5.313
10	8.233
11	12.063
12	16.407
13	20.893
14	25.310
15	29.557
16	33.617
17	37.483
18	41.15
20	48.003

The NMR spectra were obtained in a Bruker DPX 200 spectrophotometer with Quattro Nucleus Probe (5 mm), Avance III - multinuclear probes with direct (Broadband Observe - BBO) and inverse (Broadband Inverse - BBI) observation (5 mm) - and Avance III HD - multinuclear probe Quattro Resonance Inverse - QXI (5 mm), operating at 4.7, 9.4 and 14.1 Tesla and observing hydrogen nucleus at 200, 400 and 600 MHz, and carbon nucleus at 13 to 50, 100 and 150 MHz, respectively. The samples were solubilized in deuterated chloroform ($CDCl_3$) containing tetramethylsilane (TMS) and a silver foil. The chemical shifts were reported in ppm (δ) and referenced to TMS (0.00 ppm).

4.4 RESULTS

4.4.1 ENDOPHYTES IDENTIFICATION

The fungal genera identified in the study were*: Alternaria* (LGMF626 and LGMF692) (FIGURE 2)*, Bjerkandera* (LGMF713) (FIGURE 3)*, Colletotrichum* (LGMF682) (FIGURE 4)*, Diaporthe* (LGMF625, LGMF627, LGMF651, LGMF653, LGMF655, LGMF657, LGMF658, LGMF694, LGMF695, LGMF700, LGMF701 and LGMF714), *Penicillium* (LGMF698) (FIGURE 5) and *Xylaria* (LGMF673) (FIGURE 6). It was not possible to identify the fungus LGMF659 (FIGURE 7) by the employed techniques.

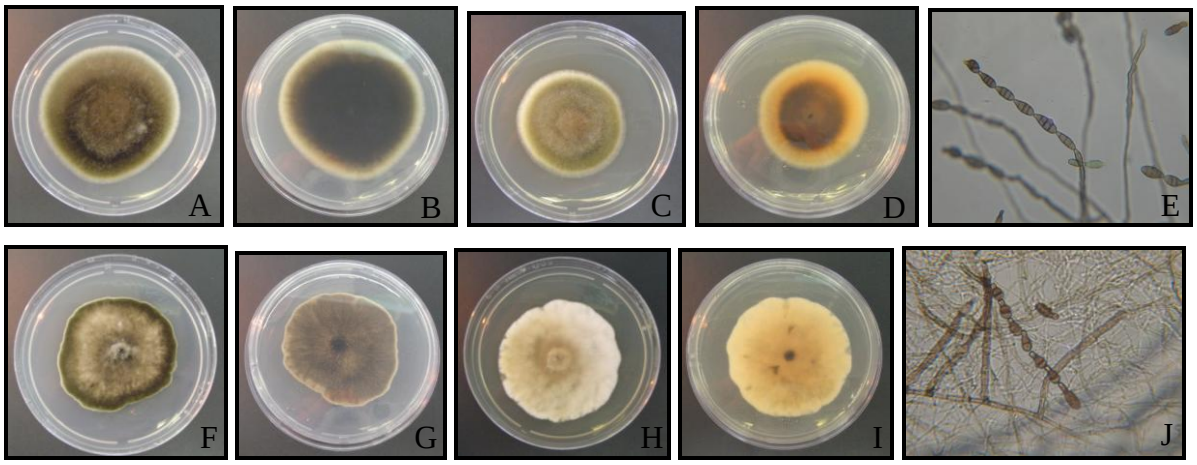


FIGURE 2. Morphological characterization of *Alternaria* sp. endophyte from leaves of *Schinus terebinthifolius.*
A **=** LGMF626 BDA-verse; B = LGMF626 BDA-reverse; C = LGMF626 MEA-verse; D = LGMF626 MEA-reverse; E = LGMF626 conidia observed on microcultive (400X); F = LGMF692 BDA-verse; G = LGMF692 BDA-reverse; H = LGMF692 MEA-verse; I = LGMF692 MEA-reverse; J = LGMF692 conidia observed on microcultive (400X).

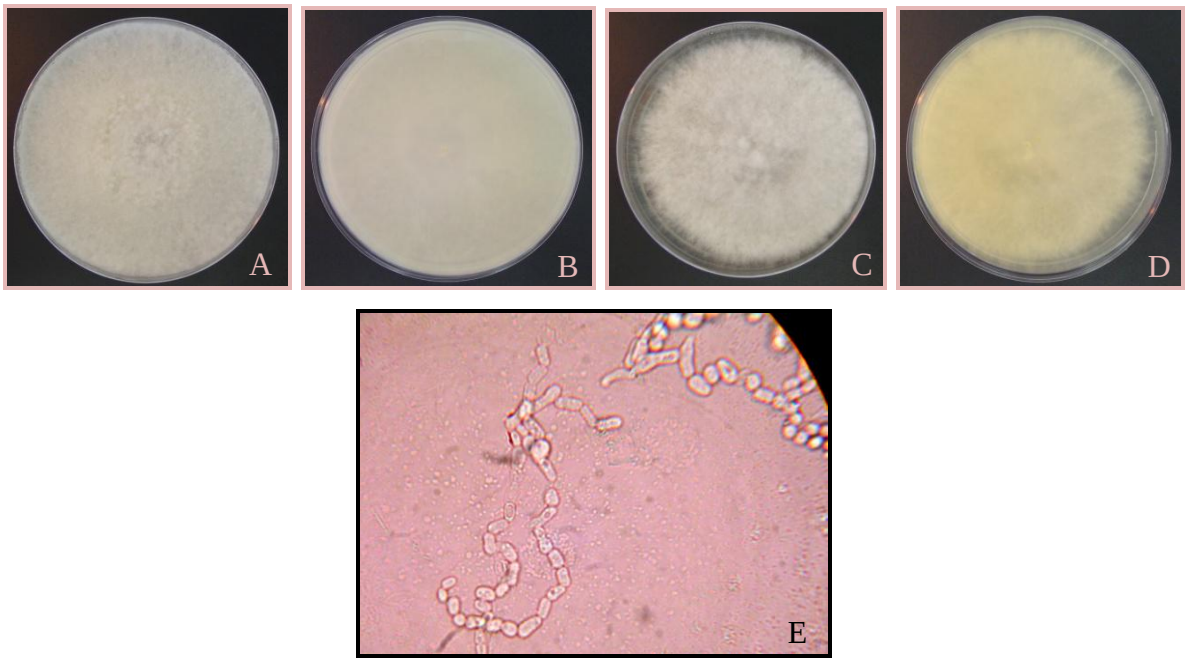


FIGURE 3. Morphological characterization of *Bjerkandera* sp. endophyte from leaves of *Schinus terebinthifolius.*
A **=** LGMF713 BDA-verse; B = LGMF713 BDA-reverse; C = LGMF713 MEA-verse; D = LGMF713 MEA-reverse; E = LGMF713 arthroconidia observed on microcultive (400X).

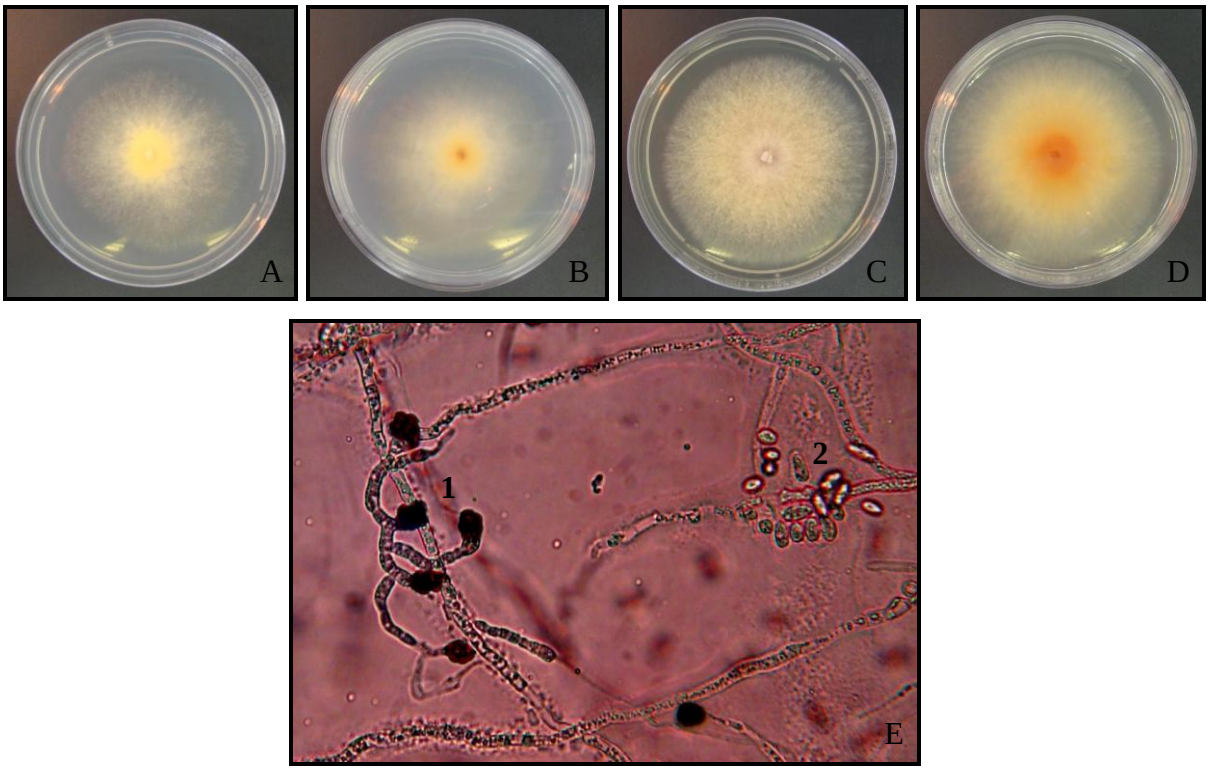


FIGURE 4. Morphological characterization of *Colletotrichum* sp. endophyte from leaves of *Schinus terebinthifolius.*
A **=** LGMF682 BDA-verse; B = LGMF682 BDA-reverse; C = LGMF682 MEA-verse; D = LGMF682 MEA-reverse; E = LGMF682 appressoria (1) and conidia (2) observed on microcultive (400X).

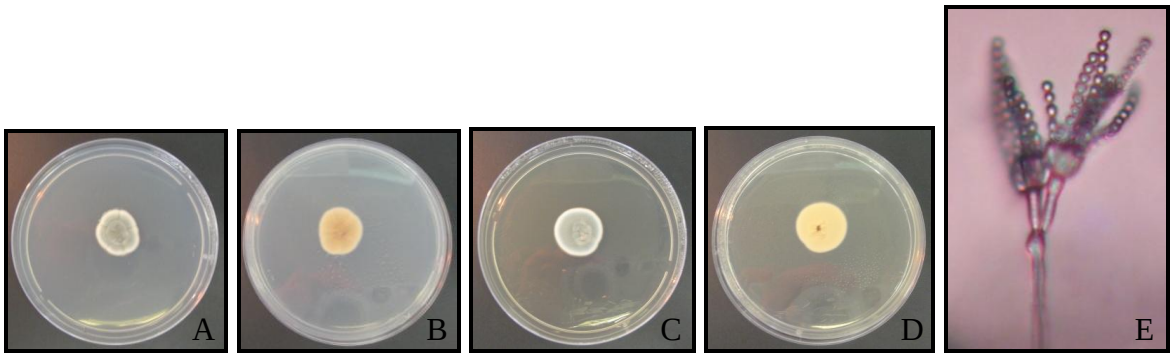


FIGURE 5. Morphological characterization of *Penicillium* sp. endophyte from leaves of *Schinus terebinthifolius.*
A **=** LGMF698 BDA-verse; B = LGMF698 BDA-reverse; C = LGMF698 MEA-verse; D = LGMF698 MEA-reverse; E = LGMF698 conidiophores, phialides and conidia observed on microcultive (400X).

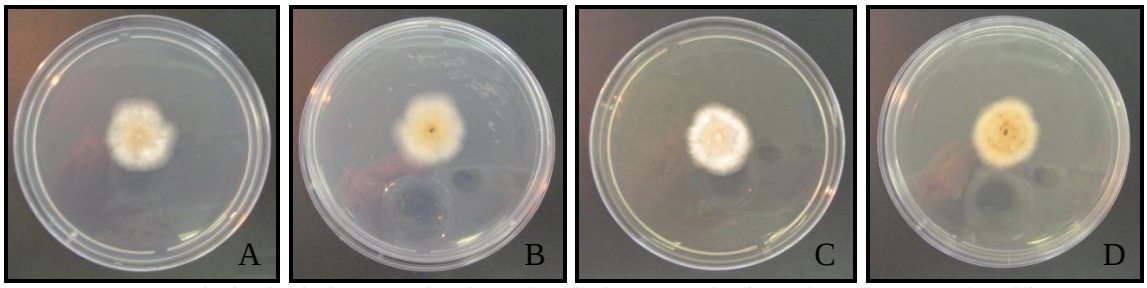


FIGURE 6. Morphological characterization of *Xylaria* sp. endophyte from leaves of *Schinus terebinthifolius.*
A **=** LGMF673 BDA-verse; B = LGMF673 BDA-reverse; C = LGMF673 MEA-verse; D = LGMF673 MEA-reverse.

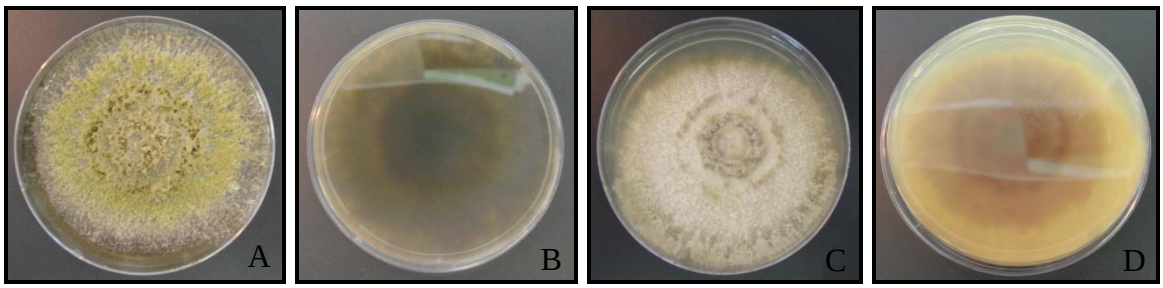


FIGURE 7. Not identified endophyte from leaves of *Schinus terebinthifolius.*
A **=** LGMF659 BDA-verse; B = LGMF659 BDA-reverse; C = LGMF659 MEA-verse; D = LGMF659 MEA-reverse.

The probable species of *Diaporthe* identified were: *D. terebinthifolii* - LGMF625, LGMF651, LGMF655, LGMF657 and LGMF658 (FIGURA 8); *D. endophytica* - LGMF653 (FIGURA 9); *D. helianthi* - LGMF694 (FIGURA 10); *D. infecunda* - LGMF627, LGMF700 and LGMF701 (FIGURA 11); and *Diaporthe* sp. - LGMF695 and LGMF714 (FIGURA 12).

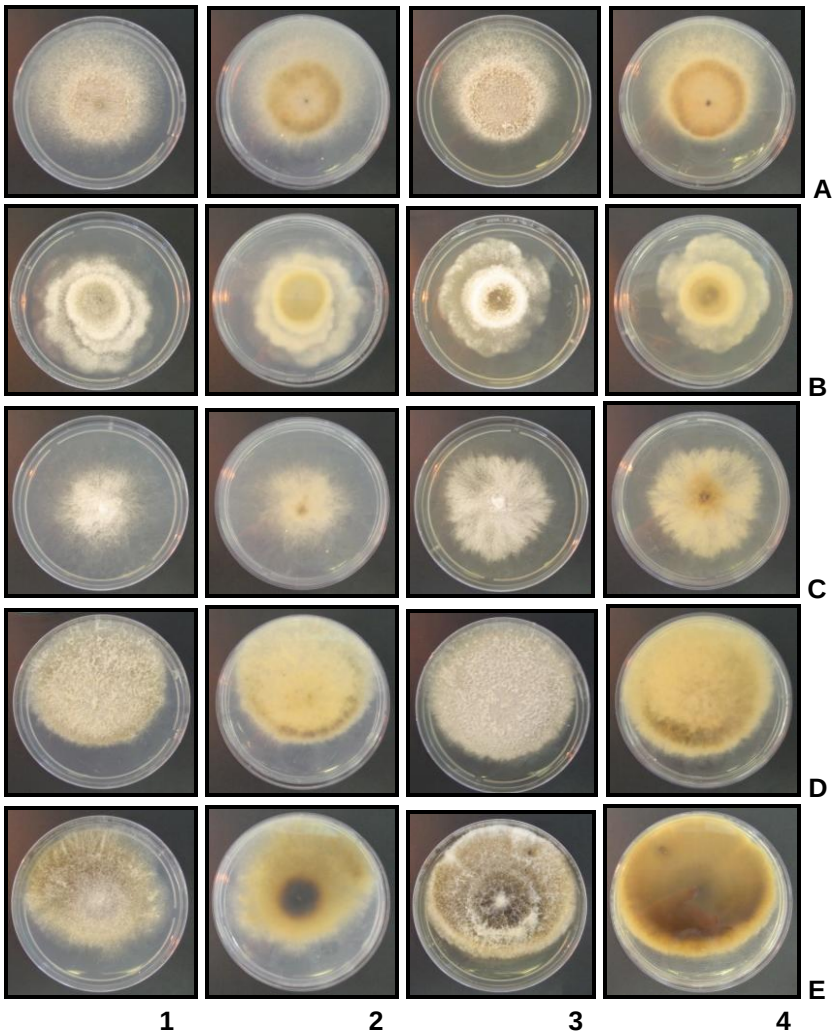


FIGURE 8. Macromorphology of five isolates of *Diaporthe terebinthifolii*
A = LGMF625; B= LGMF651; C = LGMF655; D = LGMF657; E = LGMF658.
1 = BDA-verse; 2 = BDA-reverse; 3 = MEA-verse; 4 = MEA-reverse.

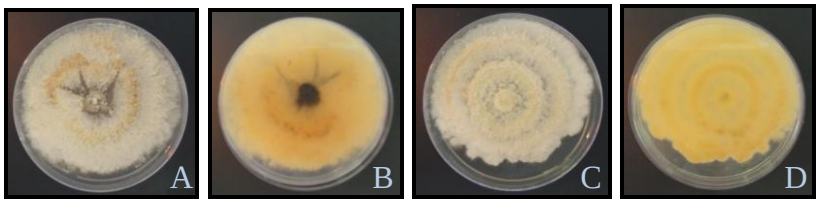


FIGURE 9. Macromorphology of one isolate of *Diaporthe endophytica*
A **=** LGMF653 BDA-verse; B = LGMF653 BDA-reverse; C = LGMF653 MEA-verse; D = LGMF653 MEA-reverse.

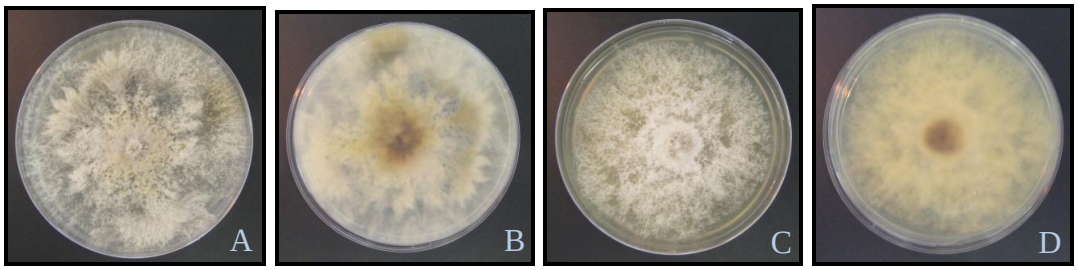


FIGURE 10. Macromorphology of one isolate of *Diaporthe helianthi*
A **=** LGMF694 BDA-verse; B = LGMF694 BDA-reverse; C = LGMF694 MEA-verse; D = LGMF694 MEA-reverse.

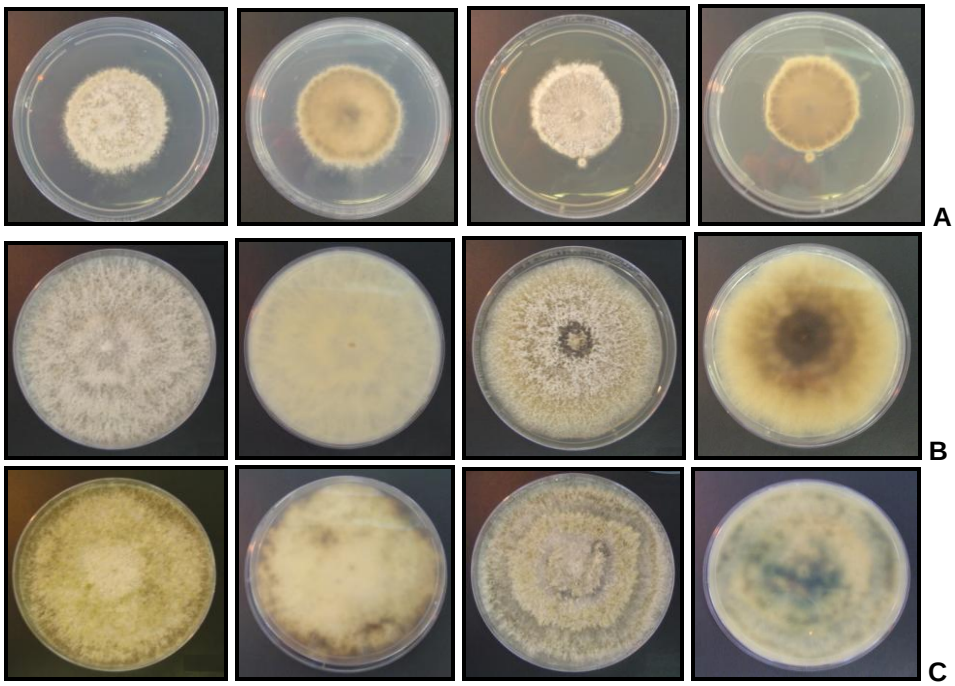


FIGURE 11. Macromorphology of three isolates of *Diaporthe infecunda*
A = LGMF627; B= LGMF700; C = LGMF701.
1 = BDA-verse; 2 = BDA-reverse; 3 = MEA-verse; 4 = MEA-reverse.

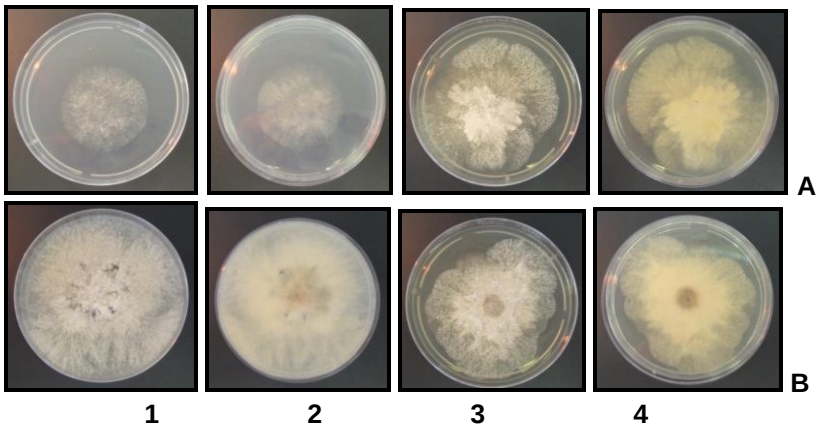


FIGURE 12. Macromorphology of two isolates of *Diaporthe* sp.
A = LGMF695; B= LGMF714.
1 = BDA-verse; 2 = BDA-reverse; 3 = MEA-verse; 4 = MEA-reverse.

The fungi LGMF626 and LGMF692, identified as *Alternaria* sp., were included in the section *alternata* (FIGURE 13).

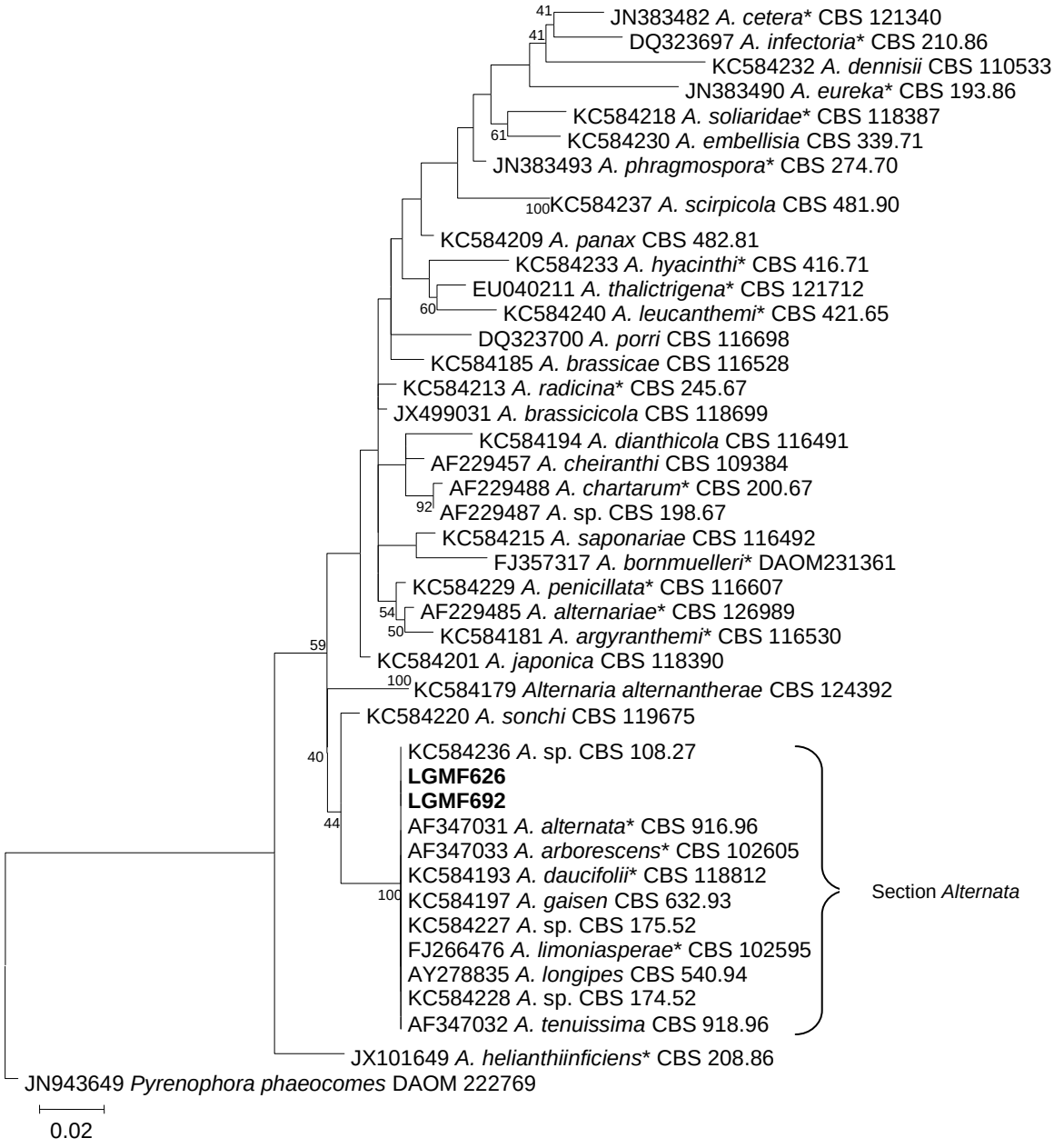


FIGURE 13. Phylogenetic tree based on alignment of *Alternaria* species including ITS1-5.8S-ITS2 of rDNA gene sequencing data. The data matrix had 42 taxa and 492 characters. *Pyrenophora phaeocomes* was used as outgroup. The number of bootstrap replications was 5000. The codes of the studied fungi are printed in bold (LGMF626 and LGMF692).
*ex-type strain

4.4.2 ANTIMICROBIAL ACTIVITY

After the evaluation of 228 extracts, it was verified that eleven fungi produced active metabolites against at least one tested pathogen. The majority of active extracts had effect on the *S. aureus*. Two fungi produced active extracts against the *P. aeruginosa* (*Bjerkandera* sp. - LGMF713 and *Diaporthe* sp. - LGMF694). Four showed activity against *C. albicans* (*Penicillium* sp. - LGMF698, *Diaporthe* sp. - LGMF627 and LGMF653, and *Xylaria* sp. - LGMF673) (TABLE 1).

The *Diaporthe* sp. - LGMF625, LGMF651, LGMF658, LGMF695, LGMF700 and LGMF714, the *Colletotrichum* sp. - LGMF682, and the isolate LGMF659 did not produced active extracts in the conditions provided.

From the nineteen isolates two of them (*Diaporthe* sp. - LGMF655 and *Alternaria* sp. - LGMF692) released bioactive compounds against *S. aureus* in MPE culture medium. After one day of cultive the *Diaporthe* sp. (LGMF655) extract obtained from the culture medium already presented active metabolites; for the *Alternaria* sp. (LGMF692) the extract bioactivity was evidenced after one week of cultive.

Ten isolates had secondary metabolites with antimicrobial activity retained on their cell structures: *Bjerkandera* sp. - LGMF713 in corn medium for one week and for one month and in Czapeck for one day; *Penicillium* sp. - LGMF698 in MPE and Czapeck for one day; *Alternaria* sp. - LGMF692 in MPE for one week; *Alternaria* sp. - LGMF626 in rice medium and Czapeck for one week; *Diaporthe* sp. - LGMF694 in Czapeck for one week; *Diaporthe* sp. - LGMF653, LGMF657 and LGMF701 in rice for one week; *Xylaria* sp. - LGMF673 in Czapeck and MPE for one day and Czapeck for one week; and *Diaporthe* sp. - LGMF627 in Czapeck for one day (TABLE 1).

There was no correlation between the probable species of the genus *Diaporthe* identified with the production of extracts with antimicrobial activity.

TABLE 1**.** Identification (ITS*), isolated, collector, GenBank Accession Number and activity (source and spectrum of the extract) of *Schinus terebinthifolius* endophytic fungi

Species	Strain	Collector	GenBank Accession Number	Active extract	Spectrum
***Alternaria* sp. Section Alternata**	**LGMF626**	**Lima JS**	**KM510497**	**RW/CWM**	***S. aureus***
	LGMF692	Tonial F	KM510498	MWF/MWM	*S. aureus*
***Bjerkandera* sp.**	**LGMF713**	**Lima JS**	**KM510499**	**CW/CM/CDM**	***S. aureus* and *P. aeruginosa***
***Xylaria* sp.**	**LGMF673**	**Lima JS**	**KM510500**	**MDM/CDM/CWM**	***S. aureus* and *C. albicans***
Colletotrichum sp.	LGMF682	Lima JS	KM510501	-	-
Diaporthe sp.	LGMF627	Lima JS	KM510503	CDM	*C. albicans*
	LGMF653	Lima JS	KM510508	RW	*C. albicans*
	LGMF655	Lima JS	KM510505	MDF	*S. aureus*
	LGMF657	Lima JS	KM510509	RW	*S. aureus*
	LGMF694	Tonial F	KM510507	CWM	*S. aureus* and *P. aeruginosa*
	LGMF701	Tonial F	KM510512	RW	*S. aureus*
	LGMF625	Lima JS	KM510502	-	-
	LGMF651	Lima JS	KM510504	-	-
	LGMF658	Lima JS	KM510506	-	-
	LGMF695	Tonial F	KM510510	-	-
	LGMF700	Tonial F	KM510511	-	-
	LGMF714	Tonial F	-	-	-
Penicillium sp.	LGMF698	Tonial F	KM510513	MDM/CDM	*S. aureus* and *C. albicans*
Not identified	LGMF659	Lima JS	-	-	-

Legend: RW - rice one week/ CWM - Czapeck one day mycelium/ MWF - MPE one week mycelium/ MWM - MPE one week mycelium/ CW - corn one week/ CM - corn one month/ CDM - Czapeck one day mycelium/ MDF - MPE one day filtrate/ MDM - MPE one day mycelium
*ITS - ITS1-5.8S-ITS2 rDNA.
In bold – isolates used for conditions optimization

4.4.3 CULTURE CONDITIONS OPTIMIZATION

The isolates *Alternaria* sp. - LGMF626, *Xylaria* sp. - LGMF673 and *Bjerkandera* sp. - LGMF713 had the culture conditions optimized. During the optimization it was not observed the retention of active compounds in the mycelium and all active extracts were obtained from the culture medium.

There was no expressive variation in the action intensity against *S. aureus* of the extracts obtained from the *Alternaria* sp. - LGMF626 cultivation with different carbon sources. However, changes in the nitrogen source increased the intensity of the activity mainly by addition of soy flour and ammonium sulfate. The optimal period of incubation varied according to the carbon or nitrogen source introduced. The acidification of the initial pH and the temperature elevation increased the activity spectrum to *C. albicans* (TABLE 2).

For *Xylaria* sp. - LGMF673 few conditions provide active extracts. The best conditions were found using sucrose and soy flour. As well as for *Alternaria* sp. - LGMF626, acidification allowed the production of active extracts against *C. albicans*. The temperature elevation had a strong influence on the intensity of action against *S. aureus* (TABLE 2).

To *Bjerkandera* sp. - LGMF713 the change in the carbon source and the incubation time were the factors that allowed the expansion of the activity spectrum of the extract. The production of active compounds against *C. albicans* by this fungus seems to be linked to the carbon source used. No extract obtained in media containing glucose produced compounds against the yeast. But this occurred in media with sucrose or galactose, being more prominent for galactose. The yeast extract as nitrogen source exerted a positive influence on the activity. The changes in pH and temperature did not improve the production of active compounds (TABLE 2).

TABLE 2. Antimicrobial activity of *Schinus terebinthifolius* endophytic fungi extracts obtained in different culture conditions by bioautography

Conditions		Carbon source									Nitrogen source									pH**		Temperature**	
		Glucose*			Sucrose			Galactose			Soy flour			Yeast Extract			Ammonium Sulfate			3.0	5.0	25 ºC	35 ºC
Isolates	Days	1	7	14	1	7	14	1	7	14	1	7	14	1	7	14	1	7	14				
Alternaria sp. - LGMF626	*S. aureus*	+++	+++	+	+	++	++	+++	+++	++	++	++++	++	-	++	+++	+++	++++	+++	++	+++	+	+++
	C. albicans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	+++	-	+
Bjerkandera sp.- LGMF713	*S. aureus*	++	++	+	+	++	++	++	+++	+	++	-	+	+	+++	+++	-	-	+	-	-	+	-
	C. albicans	-	-	-	+	-	-	++++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylaria sp. - LGMF673	*S. aureus*	-	-	+	++	-	-	+	-	-	-	++	-	-	-	-	-	-	-	-	-	-	++++
	C. albicans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	+	-	-

(-) no activity; (+) low activity; (++) moderate activity; (+++) high activity; (++++) very high activity

The positive controls resulted in (++++) for *C. albicans* and (+++) for *S. aureus*

For all conditions, no activity (-) was observed for the extracts obtained with the medium without inoculum and for others negative controls (methanol and saline solution)

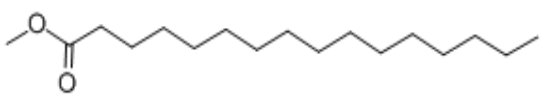
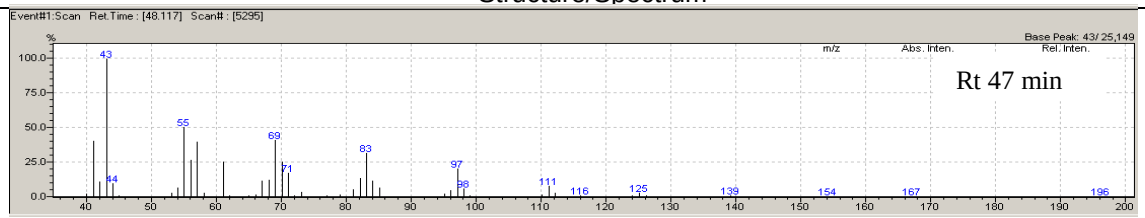
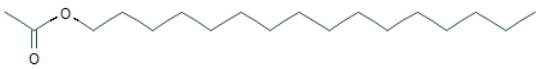
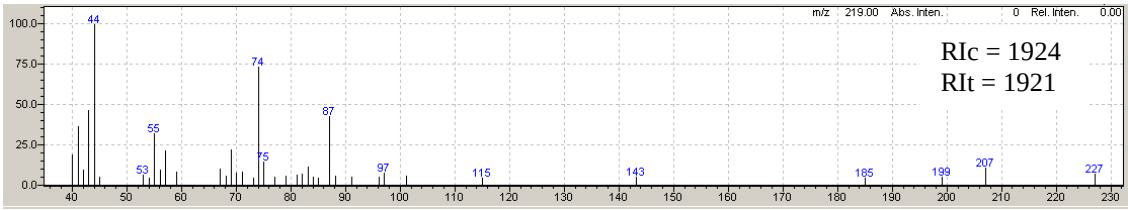
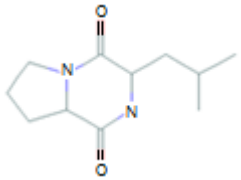
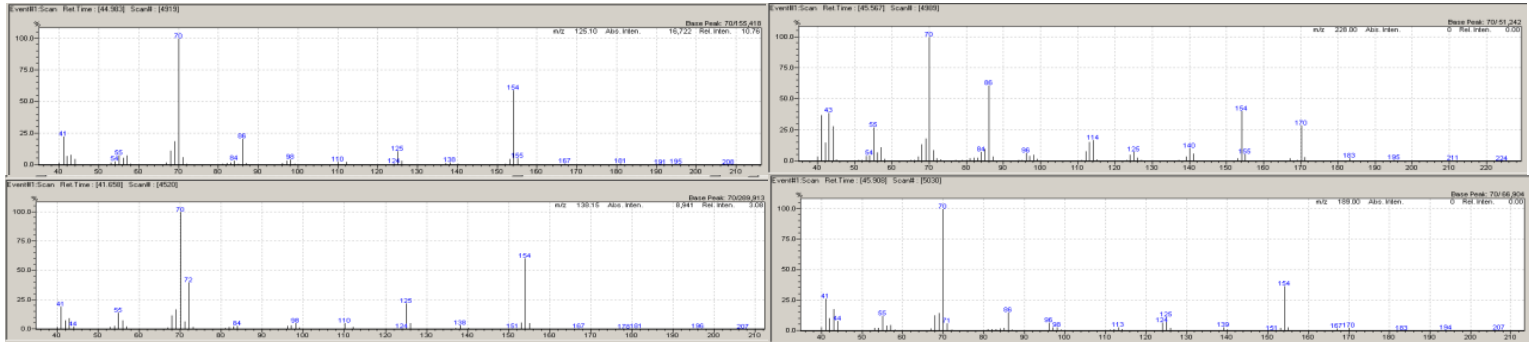
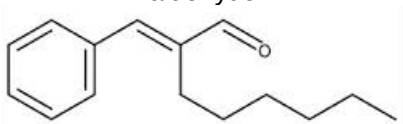
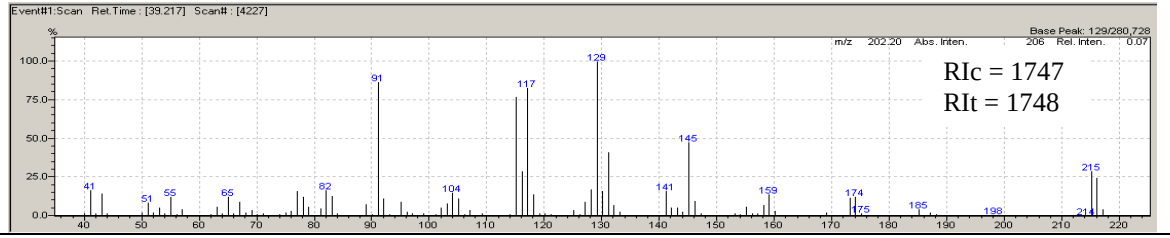
*The glucose results also include the conditions: sodium nitrate, pH 8.5 and 28 ºC

** pH and temperature are analysed in Czapeck medium with the ideal period of cultive for the isolated. pH condition was evaluated at 28 ºC

4.4.4 CHEMICAL COMPOSITION

The *Alternaria* sp.- LGMF626 produced 0.17 g of extract in 6 l of the optimized culture (glucose, ammonium sulfate, pH 5.5, 35 ºC, and 7 days in stationary conditions). The extract was fractionated on 27 parts by column chromatography. Two fractions were active against *S. aureus*; VII (8 mg) and XVIII (22 mg). HPLC analysis revealed two major components in each active fraction. In the active extract of *Alternaria* sp. - LGMF626 were detected by GC-MS the compounds: methyl hexadecanoate, hexadecyl acetate, a pyrrolopyrazine alkaloid, and E-2-hexyl cinnamic aldehyde (the last one was confirmed by NMR) (TABLE 3).

TABLE 3. Compounds, structures and gas chromatography–mass spectrometry (GC-MS) spectrums of the metabolites identified in *Alternaria* sp. - LGMF626 active extract

Compound	Structure/Spectrum
Methyl Hexadecanoate	 
Hexadecyl acetate	 
Pyrrolopyrazine alkaloid	 
E-2-Hexyl cinnamic aldehyde	 

Rt - Retention time; RIc - Retention index calculated; RIt - Retention index teorical

4.4.5 MINIMUM INHIBITORY CONCENTRATION (MIC)

The MIC of fraction VII against *S. aureus* was 18.52 µg/mL; for the fraction XVIII was 55.55 µg/mL. The MIC was the same for the ATCC strain (not resistent) and for the clinical isolate (MRSA).

4.5 DISCUSSION

Morphological characteristics and ribosomal DNA Internal Transcribed Spacer sequence analysis are still considered valuable source of evidence to resolve phylogenetic relationships for endophytic fungi (NAIR and PADMAVATHY, 2014), statement that we agree when considering just the gender level. Most of genera identified in this study are generally isolated as endophytic fungi. All of them have been recently described as bioactive metabolites producers: *Alternaria* (*SHAABAN et al*., 2012)*, Bjerkandera* (LEVY *et al*., 2000)*, Diaporthe* (LIM *et al.,* 2010; SEBASTIANES *et al*., 2012), *Penicillium* (SUPAPHON *et al.*, 2013) and *Xylaria* (RATNAWEERA *et al.*, 2014). In this study, of the fungal genera isolated, only the *Colletotrichum* was not able to produce antimicrobial compounds under the provided conditions, even though there are reports that describe endophytic isolates of this genus as producers of metabolites of interest, including against MRSA (ARIVUDAINAMB *et al*., 2011; LU *et al*., 2000). In both studies, the active extracts produced by *Colletotrichum* were obtained on potato dextrose, a culture medium not used in this work, suggesting that growing conditions were determinant in limiting the activity of our extracts for this genera. The fungi LGMF626 and LGMF692 identified as *Alternaria* sp. were included in the section *alternata* after phylogenetic analyses of ITS sequence following the work that redefined the genus (WOUDENBERG *et al*., 2013). According to Woudenberg *et al.* (2013), section *alternata* comprises almost 60 *Alternaria* species based on ITS sequence data, and the molecular variation within this section is low.

The fact that several isolates selected by macroscopic characteristics and positivity in pairing trials (Tonial, 2010) were posteriorly identified as *Diaporthe* sp. demonstrates the macromorphological diversity and biotechnological importance of the gender, which is probably not related to the species.

From 19 fungi selected, 228 extracts were tested. Eleven isolates (57.89%) produced active compounds in at least one of the production processes. All variations in culture conditions (time, medium and extraction) had influence on the obtainment of the bioactive metabolite. The production of active extracts occurred preferentially in Czapeck medium. Other studies obtained similar percentages of production of antimicrobial extracts by pre-selected fungi: 46.4% (CUI *et al.,* 2011), 47.6% (DING *et al.,* 2013) and 61.3% (BUATONG *et al.,* 2011). It is possible to assume that submission of microorganisms to other growing conditions and extraction would expand the percentage of fungi producers of active extracts, however, the demand of time and resources for such bioprospecting studies would make it impracticable.

The production of secondary metabolites by microorganisms is a specific characteristic of the strain, which depends on the growing conditions provided. This approach, that intends to increase the chances of finding new metabolites from a single strain, is known as “one strain many compounds” (OSMAC) (ALY *et al.,* 2011). So, different media and culture conditions were analyzed to obtain the most promising extracts. The optimization process to produce an extract does not discard the necessity of culture conditions optimization to reach determinate compound. But, while the bioactive metabolite is still unknown, it is an alternative to get an extract with greater concentration of the desirable compound or with less chemical interferents which, in principle, facilitates the purification process. The general environmental factors, like the ones tested in this study, regulate the metabolite production by effects in proteins signaling, like CreA (carbon source), AreA (nitrogen) and PacC (pH) (ALY *et al.,* 2011). Steroids, xanthones, phenols, isocoumarins, perylene derivatives, quinines, furandiones, terpenoids, depsipeptides, and cytochalasines are secondary metabolites produced by endophytes known to be affected in quantity and quality by changing general environmental factors in culture (VERMA *et al.,* 2009).

In this study, the optimization of culture conditions proved to be important both for obtaining extracts with stronger activity, and for expand the spectrum of them. In this step Czapeck medium was used because it produced the most active extracts in previous assays and allows change in composition, requirement for optimization. The active compounds production in most of the tested conditions by *Alternaria* sp. - LGMF626 and *Bjerkandera* sp. - LGMF713 demonstrated a stability in the process,

and consequently the importance of these metabolites for these fungi. However, for the isolate *Xylaria* sp. - LGMF673 the conditions to produce an active extract were quite restricted. In general galactose was the best carbon source influencing the action intensity and spectrum of the extracts. The medium acidification was the condition that provided the best results in terms of activity against the yeast. The best source of nitrogen and temperature were specific for each isolate.

El-Banna (2005) only evaluated the variation of the carbon source on the production of antimicrobial substances produced by various *Bacillus* isolates and demonstrates very clearly the influence of the optimization in the production of biocompounds and how the needs of each isolate are individualized. Also, just the change of the carbon source modified the type of antimicrobial compound produced (glycopeptide or lipopeptide) by a *Nocardioides* sp. (GESHEVA and VASILEVA-TONKOVA, 2012). The individualization of nutritional needs is also confirmed by comparing the results of this study with those obtained by Mitra *et al.* (2011). While in the present study we observed that the galactose favored the intensity and spectrum of action of the extracts Mitra *et al.* (2011) reported that these conditions were unfavorable for the entire microbial diversity analyzed.

It is interesting that in the first step of extracts production only 2 (10.53%) of the active extracts were obtained from the fermentation broth, the majority (89.47%) of the active compounds were obtained by maceration of cellular structures. But, after optimization, despite the use of the same media and manner of extracting, in none of the cultivations the retention of active compounds in the mycelium was verified. It is believed that such change in storage of metabolites is due to the aeration of medium. It is known that fungi have alternative routes to the oxidation of NADH, which consequently when alternated influences other metabolic cycles (ESPOSITO and AZEVEDO, 2010). We observed that the stress produced by the oxygen reduction dissolved in the medium (because of the increase in medium volume and absence of shaking), induced the release of active secondary metabolites in the medium. The use of two different methodologies to the evaluation of initial extracts, bioautography and method of wells, it has become necessary due to low yield of the extracts obtained from the filtered (data not shown). The method of wells is more economical, but the bioautography utilizes a fifth of volume of extract in each sampling. Both methods are widely used in bioprospecting of secondary metabolites with antimicrobial activity. So, we do not believe that the superior results

obtained by macerating the mycelia in the first step of production have occurred by the sensitivity of the technique (wells method), but by the characteristics of cultivation. Also because, during the optimization results obtained were the inverse, bioautography showed the positives results while the wells method did not. The polarity of the solvent for extraction can not be considered the reason for the reversal of results, because the extraction methodology for the two steps was identical.

Regarding the MIC for *S. aureus* of the fractions produced in large scale (*Alternaria* sp. - LGMF626), the antimicrobial activity detected may be considered high (VII - MIC = 18.52 µg/ml) and moderate (XVIII - MIC = 55.55 µg/ml) if compared with the *Alternaria* spp. compounds described below. It also calls attention the fact that they were identical to the clinical isolate resistant to methicillin and the susceptible strain. Therefore, the mechanism of action of active metabolites is not influenced by β-lactamase presented by the MRSA clinical isolate. Secondary metabolites with activity against MRSA obtained from the genus *Alternaria* spp. have been previously described. Among them, altenusin with MIC of 31.25 µg /mL against MRSA (KJER *et al.,* 2009) and MIC of 25 µg /mL against a not resistant *S. aureus* (XU *et al.,* 2012) and xanalteric acids I and II, with low activity against the resistant strain (MIC of 125 and 250 µg /mL, respectively) (KJER *et al.,* 2009). The altenusina also showed activity against *Streptococcus pneumoniae* (KJER *et al.,* 2009) and significant antifungal activity against clinical isolates of *Paracoccidioides brasiliensis* with MIC between 1.9 and 31.2 µg /mL (JOHANN *et al.,* 2012).

Among the compounds identified - methyl hexadecanoate, hexadecyl acetate, pyrrolopyrazine alkaloid, and E-2-hexyl cinnamic aldehyde - it is probable that the antimicrobial activity observed in this study is related to the methyl hexadecanoate or to the pyrrolopyrazine alkaloid by these have already reported bioactivity. No studies were found relating the production of these compounds to the genus *Alternaria*.

The methyl hexadecanoate (a palmitate - ester of palmitic acid) has already been isolated as a secondary metabolite from an endophytic actinomycete of tomato plant, being considered one of the acaricides compounds from the endophyte extract (CHEN *et al.,* 2011); its acaricidal activity was further studied by the same research group and suggests the neurotoxic action of the compound on mites, as well as the autolysis of structural membranes (WANG *et al.,* 2010). The compound was also detected in a mixture of volatile metabolites obtained from the endophytic bacterium

Burkholderia tropica, which presented activity against phytopathogenic fungi (TENORIO-SALGADO *et al.,* 2013). One study suggests that the methyl hexadecanoate, with the synonymous of methyl ester hexadecanoic acid, is directly involved with the anti-inflammatory and antimicrobial activity of essential oils from *Perovskia abrotanoides* leaves (ASHRAF *et al.,* 2014). It is also part of the essential oil composition from *Myrica esculenta* stem bark, which has potent antimicrobial and significant anti-inflammatory activities (AGNIHOTRI *et al.,* 2012). It is confirmed the anti-inflammatory activity (EL-DEMERDASH, 2011; SAEED *et al.,* 2012), it protects against the formation of lung fibrosis (RODRÍGUEZ-RIVERA *et al.,* 2008; EL-DEMERDASH, 2011; MANTAWY *et al.,* 2012; SHARAWY *et al.,* 2013), it stimulates the proliferation of mesenchymal cells from bone marrow of mice (ZENG *et al.,* 2008), is an inhibitor of phagocytosis by macrophages (CAI *et al.,* 2005; SARKAR *et al.,* 2006), and is vasodilator (LIN *et al.,* 2008; LEE *et al.,* 2010).

Pyrrolopyrazine alkaloids are bicyclic heterocycle derived from the condensation of a pyrole with a pyrazine, however, are apparently poorly studied due to the limited number of synthesis methods (PARJANE *et al.,* 2010). The antimicrobial activity of this class of alkaloids has been highlighted in extracts of endophytic fungus *Mortierella alpina* containing the metabolites pyrrolo-[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) as major components (MELO *et al.,* 2014). Other endophytes produce pyrrolopyrazine type secondary metabolites: *Acremonium lolii* (ROWAN *et al.,* 1990; ROWAN, 1993), *Epichloë/Neotyphodium* (TANAKA *et al.,* 2005). The fish pathogens *Acinetobacter* sp. and *Vibrio parahaemolyticus* also produced actives extracts against Gram-negative bacteria containing pyrrolopyrazine (PANDEY *et al.,* 2010; PANDEY *et al.,* 2011).

Other compounds isolated from *Alternaria* spp. with antibacterial activity but not found on LGMF626 extract include: porric acid D with MIC of 100 µg /mL against *S. aureus* (XU *et al.,* 2012); altersetin with strong activity against Gram-positive pathogens (HELLWIG *et al.,* 2002); bassicicolin A with potent activity against *S. aureus* and *Bacillus subtilis* (GLOER *et al.,* 1988); altersolanol and two derivatives active against Gram positive bacteria and *Pseudomonas aeruginosa* (OKAMURA *et al.,* 1993; YAGI *et al.,* 1993); altersolanol C, macrosporin and alterporriol C with potent activity against *Escherichia coli* and *Vibrio parahemolyticus* (ZHENG *et al.,* 2012); alternaramide with poor biological activity against *B. subtilis* and *S. aureus*

(KIM *et al.,* 2009); three alternarosides (A-C) and a alternarosin showed weak activity against *E. coli* and *B. subtilis* (WANG *et al.,* 2009); tenuazonic acid active against *Mycobacterium tuberculosis* (MIC 250 µg /mL) (SONAIMUTHU *et al.,* 2011); and altechromone that presented MICs of 3.9, 3.9 and 1.8 µg /mL against *B. subtilis, E. coli* and *Pseudomonas fluorescens*, respectively (GU, 2009).

The antimicrobial activity, in general terms, increases the variety of interesting compounds isolated from the genus *Alternaria*. Antifungal activity against *Candida albicans* was observed for some compounds previously reported, the three alternarosides (A-C), the alternarosin (WANG *et al.,* 2009) and the altechromone (GU, 2009). The herbarin A was active against *Trichophyton rubrum* and *C. albicans* with MICs of 15.6 µg /mL for both (GU, 2009). The pyrophen and rubrofusarin showed high (28 mm) and moderate (12 mm) activity against *C. albicans*, and the auraspenore was active (13 mm) against the phytopathogenic fungus *Rhizoctonia solani* (SHAABAN *et al.,* 2012). Cyclo-[L-Leu-trans-4-hydroxy-L-Pro-], cyclo-[L-Phe-trans-4-hydroxy-L-Pro-], and cyclo-[L-Ala-trans-4-hydroxy-L-Pro-] inhibited the sporulation of the phytopathogen *Plasmopara viticola* (Musetti *et al.* 2007). The fumitremorgin B, the fumitremorgin C and the helvolic acid, known for its important antimicrobial action, significantly inhibited the growth of many phytopathogens; another compound isolated in the same study, the cyclo-(Phe-Ser) showed moderate activity against the same pathogens (CHENGLIANG and YANGMIN, 2010). Alterperylenol and dihydroalterperylenol showed activity against the phytopathogen *Valsa ceratosperma* (OKUNO *et al.,* 1983).

Including antiviral activity we still have the compounds tetrahydroaltersolanol C, alterporriol Q and alterporriol C active against a swine virus (ZHENG *et al.,* 2012); alternariol and alternariol monomethyl ether with activity against herpes virus (HE *et al.,* 2012); and a compound belonging to the class of natural compounds known as perylenequinones active against HIV-1 (WELLENSIEK *et al.,* 2013). The reported compounds belong to a variety of chemical classes emphasizing the complexity and diversity in the secondary metabolism of a single fungal genus.

The *Alternaria* species that were identified as producers of bioactive metabolites were: *A. tenuissima* responsible for producing the perylenequinones (WELLENSIEK *et al.,* 2013); *A. alternata* as the producer of pyrophen, rubrofusarin, aurosperone (SHAABAN *et al.,* 2012), alternariol, alternariol monomethyl ether (HE *et al.,* 2012), cyclo-[L-Leu-trans-4-hydroxy-L-Pro-], cyclo-[L-Phe-trans-4-hydroxy-L-

Pro-], cyclo-[L-Ala-trans-4-hydroxy-L-Pro-] (MUSETTI *et al.,* 2007), and tenuazonic acid (SONAIMUTHU *et al.,* 2011). According to Woudenberg *et al.* (2013) *A. tenuissima* and *A. alternata* are phylogenetically in the same section, *alternata*, in which the endophyte of aroeira studied (LGMF626) was also classified, demonstrating a high potential for production of bioactive compounds by the fungus belonging to this section.

Other *Alternaria* species that produces active compounds include: *A. raphani*, from which were extracted the alternarosides (A-C) and the alternarosin (WANG *et al.,* 2009); the brassicicolin was isolated from the *A. brassicicola* (GLOER *et al.,* 1988.), as well as altechromone and herbarin A (GU, 2009); the altersolanol and its derivatives were obtained from an isolate of *A. solani* (OKAMURA *et al.,* 1993; YAGI *et al.,* 1993). For xanalteric acid, altenusin (KJER *et al.,* 2009), alternaramide (KIM *et al.,* 2009), alterperylenol, dihydroalterperylenol (OKUNO *et al.,* 1983), altersolanol C, macrosporin, alterporriol C, tetrahydroaltersolanol C, alterporriol Q (ZHENG *et al.,* 2012), fumitremorgine B, fumitremorgine C, helvolic acid, cyclo-(Phe-Ser) (CHENGLIANG and YANGMIN, 2010), altersetin (HELLWIG *et al.,* 2002) and porric acid D (XU *et al.,* 2012) it was not identified the specie of *Alternaria* that the metabolite was extracted.

The fungi tested, showed that endophytes of *S. terebinthifolius*, a Brazilian medicinal plant, can be a promising source of antimicrobial compounds, including against MRSA, which must be further investigated.

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5 ATIVIDADE BIOLÓGICA DE EXTRATOS DE *DIAPORTHE TEREBINTHIFOLII* CONTRA *PHYLLOSTICTA CITRICARPA*

5.1 Resumo A mancha preta do citros, causada pelo fitopatógeno *Phyllosticta citricarpa*, deprecia o fruto para a comercialização *in natura*, impede a exportação para regiões que não possuem a doença, e pode reduzir a produtividade dos pomares. Na busca de uma alternativa para o controle da doença, dois isolados de *Diaporthe terebinthifolii*, provenientes de uma diversidade de gêneros fúngicos endofíticos das folhas de aroeira, foram selecionados por produzirem extratos ativos contra o fitopatógeno. Diferentes meios de cultura, sólidos e líquidos, foram utilizados para a produção dos extratos. O meio de cultura e o isolado que forneceram o extrato mais eficiente tiveram a escala de produção ampliada, na tentativa de se obter um produto de interesse. Uma fração do extrato protegeu completamente as folhas de laranjeira do ataque do fitopatógeno e apresentou uma concentração inibitória mínima de 0,003 mg/mL na inibição da germinação de conídios de *P. citricarpa*. Foram identificados, por cromatografia gasosa acoplada a espectrometria de massa, os compostos: verbanol, álcool feniletílico, acetato, verbenil e hexadecanoato de metila, os quais podem estar relacionados com a bioatividade observada. Os resultados sugerem um efeito sinérgico entre os metabólitos produzidos.

Palavras chave: *Phyllosticta citricarpa* - *Diaporthe terebinthifolli* - Sinergismo - Metabólitos secundários - Compostos orgânicos voláteis

5 BIOLOGICAL ACTIVITY OF *DIAPORTHE TEREBINTHIFOLII* EXTRACTS AGAINST *PHYLLOSTICTA CITRICARPA*

5.1 Abstract The citrus black spot, caused by the phytopathogen *Phyllosticta citricarpa,* depreciates citrus in fresh fruit market, avoid the export to regions free of the disease and may reduce productivity in fruit orchards. In search of alternatives for disease control, from a diversity of endophytic fungal genera of aroeira leaves were selected two isolates of *Diaporthe terebinthifolii* producers of active extracts against this phytopathogen. Different culture media, solid and liquid, were used in the production of extracts. The culture medium and the isolate that afforded the most efficient product had the production scale enlarged in an attempt to obtain a product of interest. A fraction of the extract completely protected orange leaves of the pathogen and presented 0.003 µg/mL as minimum inhibitory concentration against the germination of *P. citricarpa* conidia. The *D. terebinthifolii* products: verbanol, phenylethyl alcohol, verbenyl acetate and methyl hexadecanoate, identified by gas chromatography coupled to mass spectrometry, can be related to the bioactivity observed. The results strongly suggest a synergistic effect among the metabolites produced.

Keywords: *Phyllosticta citricarpa* - *Diaporthe terebinthifolli* - Synergism - Secondary metabolites - Volatile organic compounds

5.2 INTRODUCTION

Citrus black spot (CBS), first described by Benson (1895) in Australia, affects fruits and leaves of citrus cultures causing economic loss to producers (KIELY, 1948). The disease was preliminarily characterized by Cobb (1897) as a roundish cavity in the fruit with dark spots; various types of lesions have been defined (KIELY, 1948). The CBS does not cause internal decay in the fruit, hitting only the rind, but depreciates the citrus in fresh fruit market. Besides, heavy infection may lead premature fruit drop decreasing productivity (KIELY, 1948; KOTZÉ, 1981). The causal agent of CBS was identified by McAlpine (1899) as *Phoma citricarpa*, based in the structure of pycnidial form of the fungus occurring in typical lesions, being the teleomorph *Guignardia citricarpa* Kiely (1948). The anamorphous was reclassified as *Phyllosticta citricarpa* (MCALPINE, 1973; GLIENKE *et al.*, 2011).

The disease is typical in subtropical zones in the following countries: Argentina, Australia, Brazil, China, Ghana, Indonesia, Kenya, Mozambique, Philippines, South Africa, Taiwan, Uruguay, Uganda, Venezuela, Zambia, and Zimbabwe (HINCAPIE *et al.*, 2014). To prevent the introduction of *P. citricarpa* in free regions of the fungus, phytosanitary legislation in UE (EFSA, 2014) regulate the interception of infested citrus fruit during import inspections. The ability of the disease to spread during storage (COBB, 1904; MCCLEERY, 1939) also hinders the export, since apparently healthy fruits can reach the destination with black spot symptoms. Besides phytosanitary measures, in 2010, the Animal and Plant Health Inspection Service (APHIS) of USA confirmed the identification of *P. citricarpa* on citrus fruit from Florida, this is the first confirmation of CBS in this country, losing the rights to maintain phytosanitary barrier.

Cultural practices and fungicides treatments pre- and post-harvest, and physical treatments of citrus fruit are used to reduce the level of CBS in the orchard or to delay symptom development in transit and storage, once it is impossible to eradicate the disease (KOTZÉ, 1981; AGOSTINI *et al.,* 2006). Some chemical products currently used to control CBS include copper fungicides, benzimidazoles, strobilurins, mancozeb, phosphorous acid, captan, iprodione (AGOSTINI *et al.*, 2006; MILES *et al.*, 2004). However, the resistance to these compounds has already been related. The recently introduced *P. citricarpa* in Florida seems to be resistant to

copper fungicides (HENDRICKS *et al.*, 2013). In Brazil, there was reported resistance to benzimidazoles (RODRIGUES *et al.*, 2007). A South African strain has also shown resistance to a benzimidazole compound (POSSIEDE *et al.*, 2009). Beyond resistance another limitation factor to the use of fungicides for CBS control is the restriction on exports to the USA of citrus products, including orange juice, containing carbendazim, a benzimidazole. Because the use of carbendazim in food products is not approved by Food and Drug Administration's (FDA's) in the USA (FDA, 2012).

Considering that there is no treatment to eradicate CBS and the limitations in fungicides use, the search for a new chemical control is a need. An important source of new compounds is the endophytic fungi. Looking for a promising source of chemical agents against *P. citricarpa,* 128 endophytes of aroeira leaves were paired with the phytopatogen. Those with inhibition potential had the crude extracts evaluated for the presence of interesting products. Two *Diaporthe* strains were selected as most promising against *P. citricarpa. Diaporthe* spp. endophytes are known for producing compounds with antifungal activity, including against *P. citricarpa* (HUANG *et al.*, 2008; WU *et al.*, 2008; HUSSAIN *et al.*, 2011; KROHN *et al.*, 2011; HUSSAIN *et al.*, 2012; FLORES *et al.*, 2013). But, there is no study about the production of secondary metabolites for *D. terebinthifolii* since this species was recently described in Brazil (GOMES *et al.*, 2013). Then, the objective of this study is to obtain a promising extract against *P. citricarpa* produced by endophyte *D. terebinthifolii* to the future development of a product.

5.3 MATERIALS AND METHODS

5.3.1 FUNGAL MATERIAL

Two endophytic fungi collected from leaves of aroeira (*Schinus terebinthifolius*) in Brazil (GLIENKE *et al.*, 2012) and deposited at LabGeM Culture Collection (LGMF651 and LGMF658), Federal University of Parana, Brazil, were used in the study. The *P. citricarpa* strain is deposited at the same Mycotec: LGMF06.

5.3.2 ENDOPHYTES IDENTIFICATION

The species was identified based on phylogenetic taxonomy with multi sequence alignment of the following genes parts: internal transcriber spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA gene operon with the primers ITS1 and ITS4; calmodulin (CAL) with the primers CAL-228F and CAL-737R; translation elongation factor 1-α (TEF1) with the primers EF1-728F and EF1-986R; histone H3 (HIS) with the primers CYLH3F and H3-1b; and β-tubulin (TUB) with the primers T1 and Bt-2b. For DNA extraction all standard procedures of Ultra-Clean™ Microbial DNA Kit (MO Bio, Carlsbad, CA, USA) were followed. PCR amplification was performed as previously described by Gomes *et al.* (2013) in Eppendorf® Mastercycler thermal cycler Gradient model. The amplified DNA was sequenced at ABI 3130 automatic sequencer (Perkin-Elmer, Massachusetts, USA). The consensus sequences were visually inspected using MEGA 6.0 with the aid of BioEdit 7.0. Muscle software was used to the alignment of multiple sequences. For the phylogenetic analyses a Bayesian inference was done using MrBayes 3.2. The Markov Chain Monte Carlo (MCMC) analysis of four chains was started in parallel from a random tree topology and lasted until the average standard derivation od split frequencies came to 0.016 (500000 generation). The matrix was build with 55 taxa and 2488 characters. The choice of taxa included in the analysis considered: fungi with specific multi primers sequences available, originating in South America, nonpathogenic, with phylogenetic proximity. All taxa analyzed were cited by Gomes *et al.* (2013). *Diaporthella corylina* (CBS 121124) was used as outgroup.

5.3.3 FUNGI BIOLOGICAL ASSAYS

5.3.3.1 Pairing against *Phyllosticta citricarpa*

The strains (endophytes and phytopatogen) were cultured on plate of potato dextrose agar (PDA) at 28 ºC for 7-21 days. Then, a disc (10 mm) of *P. citricarpa* mycelium of the edge of colony was inoculated in the middle of a Petri dish with PDA. On the same plate, 1 cm from the edge and 3 cm from the pathogen, two disks of the endophyte were inoculated, one on each side. Five plates containing only the pathogen disc were prepared as growth control. The plates were incubated at 28 ºC

for 14 days when the diameter of the pathogen was measured in two points of the colony, disregarding the initial inoculum size. The assay was performed in quintuplicate. The percentage of inhibition was calculated with the following formula:

$$PI\% = \frac{Cd - Pd}{Cd} \times 100$$

PI = percentage of inhibition; Cd = growth control diameter; Pd = pairing diameter

5.3.4 FUNGI BIOLOGICAL ASSAYS (VOLATILE METABOLITES)

5.3.4.1 Plates overlapping

The strains (endophytes and phytopatogen) were cultured on plate of potato dextrose agar (PDA) at 28 ºC for 7-21 days. Then, one disc (6 mm) of mycelium of the edge of colony was inoculated in the middle of Petri dishes with PDA. The base of the plates containing the pathogen was overlapped to those containing the endophyte. Plastic paper (PVC) was used to keep the bases together and seal the system. Five growth control systems, a plate with one pathogen disc overlapped to other containing only PDA, was sealed by the same way described before. The plates were incubated at 28 ºC for 14 days. The diameter of the pathogen was measured in four directions of the colony at the 7 º and 14 º day, disregarding the initial inoculum size. The assay was performed in quintuplicate. The percentage of inhibition was calculated as described in pairing against *Phyllosticta citricarpa*.

5.3.4.2 Plate with a division

The strains (endophytes and phytopatogen) were cultured on plate of potato dextrose agar (PDA) at 28 ºC for 7-21 days. Then, one disc (6 mm) of mycelium of the edge of pathogen colony was inoculated in a side of the Petri dishes with PDA. Similarly, on other side of the plate, it was inoculated the endophyte disc. Plastic paper (PVC) was used to seal the system. Five plates of growth control, containing only the pathogen disc, were prepared and sealed by the same way described before. The plates were incubated at 28 ºC for 14 days. The diameter of the pathogen was measured in four directions of the colony at the 7 º and 14 º day, disregarding the initial inoculum size. The assay was performed in quintuplicate. The

percentage of inhibition was calculated as described in pairing against *Phyllosticta citricarpa*.

5.3.5 CULTIVATION

Six culture media were tested to find the most promising extract against the pathogen: potato dextrose broth (PDB) pH 5.5, Mueller-Hinton Broth pH 6.8, Czapeck pH 8.0, and MPE pH 6.5; and rice and corn solid media. The strains were cultured on plate of potato dextrose agar (PDA) at 28 ºC for 7 days. Then, six discs (10 mm) of mycelium of the edge of colony were inoculated in 600 mL of liquid media (1 L Erlenmeyer flasks) or 100 g of solid media (1 L Erlenmeyer flasks). The cultives were incubated at 28 ºC for 10-30 days.

5.3.6 METABOLITES EXTRACTION

The liquid cultives were filtrated and only the aqueous portion was extracted with ethyl acetate (twice the volume). For solid media, the compounds were extracted with methanol (twice the volume). All extracts were resuspended in methanol at a final concentration of 10 mg/mL.

5.3.7 LARGE SCALE CULTIVE AND METABOLITES IDENTIFICATION

The large scale cultive was performed just for the LGMF658 strain. The fungi was cultived for 30 days in 1600 g of rice medium and extracted with 5 L of methanol, yielding around 13 g. A portion of the dried extract (2.8 g) was resuspended in a mixture methanol: water (2:1), and then partitioned in three solvents: petroleum ether, dichloromethane and ethyl acetate. All portions were tested for growth inhibition of the pathogen on the leave. The bioactive portion (dichloromethane - 1.5 g) was subjected to column chromatography on silica gel 60 and eluted with: petroleum ether; petroleum ether: ethyl acetate (100:25; 150:50; 75:100); ethyl acetate; ethyl acetate: methanol (50:25; 50:50); methanol to yield ten fractions (fractions I-X). The active fraction against the growth of the pathogen on the leaves (VI - 48 mg) was separated in four subfractions (A-D) by HPLC (H_2O:MeOH - 70:30 -

25min). The fraction (VI) and its portions had the activity evaluated by broth microdilution. The crude extract, the active fraction (VI), the active subfraction (C) and another portion of the extract active against *C. albicans* (data not showed) were chemically analyzed by GC-MS.

5.3.8 GENERAL EXPERIMENTAL PROCEDURES

Column chromatography was carried out on silica gel 60 (70-230 mesh, Merck®), 15 cm x 3,5 cm. Thin-layer chromatography (TLC), used to monitor fractions of chromatography, was performed on silica gel 60 G/UV_{254} (Macherey-Nagel®). The visualization of the TLC plates was achieved with a ultraviolet (UV) lamp (λ_{max} 254 and 365 nm) and anisaldehyde spray reagent (acetic acid:sulfuric acid:anisaldehyde, 50:1:0,5) followed by heating. High Performance Liquid Chromatography (HPLC) analyses were performed on a Flexar (Perkin Elmer®) model, with autosampler and Photodiode Array (PDA) detector. The column used was a C_{18}, 250 x 4,6 mm, 5 µm. All HPLC solvents were of HPLC grade (Vetec®), and all other chemicals used were of analytical grade. The compounds identification was done in gas chromatography coupled to mass spectrometry (GC-MS). The methanol used to resuspend the extracts and to biological assays was obtained from Merck®. To obtain the GC-MS spectra the samples were solubilized in dichloromethane ultrapure (Jt baker®) and helium was used as a carrier gas. The peaks were compared with those in a mass spectrum library of the corresponding organic compounds.

5.3.9 BIOLOGICAL ASSAYS USING EXTRACTS

5.3.9.1 Mycelial growth inhibition

A volume of extract (10 mg/mL) of 50 µL were spread over the surface of PDA medium (48 x 12 mm plates). Then, a disc (6 mm) of *P. citricarpa* mycelium, of a recent growing, was placed at the center of the plate. The assay was incubated for 28 days at 28 ºC, each 7 days the mycelium growth was measured. To obtain the inhibition percentage of mycelial growing, the treatment means were compared with the negative control (media extract without inoculum). The positive control was glifosinate (5 mg/mL). The assay was performed in quintuplicate.

5.3.9.2 Growth inhibition of the pathogen *in vivo*

Fragments (2,0 x 1,5 cm) of healthy leaves of orange tree (*Citrus sinensis)* were washed and autoclaved (20 min; 120 ºC; 1 atm). Each leaf fragment was placed on Petri dishes with water-agar; on the leaf the phytopatogen was inoculated. Then 10 µL of treatment or control was applied over the leaf with inoculum. The plates were kept for 30 days at 28 ºC. Methanol was used as a negative control. The positive control was glifosinate (5 mg/mL). The assay was performed in quintuplicate and repeated twice. The results were reported as presence/absence of pathogen development over the leaf. The concentrations of the fractions were: 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.1 mg/mL, 0.05 mg/mL.

5.3.9.3 Broth microdilution (minimum inhibitory concentration - MIC)

In each well of a microdilution plate with 96 wells were added: 90 µL potato dextrose broth, 10 µL of the treatment being evaluated and 50 µL of a conidial suspension of *P. citricarpa* with $6x10^5$ conidia/mL. The concentration of conidia in physiological solution was performed in a Neubauer counting chamber. The assay was incubated at 28 °C for 20 days. The absence of fungal growth in the well was considered positive result. The positive control was glifosinate (10 mg/mL). The control of solvent of the compounds (methanol), the control of the growth of the pathogen and the sterility control of the medium were also prepared. The assay was performed in triplicate. A serial dilution of the compound to be evaluated was performed to determine the minimum inhibitory concentration. For the fraction (stock solution of 3.0 mg/mL) the concentrations evaluated were: 200 µg/mL, 13 µg/mL, 0.65 µg/mL, 0.04 µg/mL, 0.003 µg/mL and 0.0002 µg/mL. For the subfraction C (stock solution of 4.0 mg/mL) the concentrations evaluated were: 260 µg/mL, 17.3 µg/mL, 1.15 µg/mL, 0.08 µg/mL, 0.005 µg/mL and 0.0003 µg/mL. For the positive control (glifosinate 10 mg/mL): 666 µg/mL, 44.4 µg/mL, 2.96 µg/mL, 0.2 µg/mL and 0.013 µg/mL.

5.4 RESULTS

5.4.1 ENDOPHYTES IDENTIFICATION

The search for a new alternative to control Citrus Black Spot disease led to the identification of two strains among a diversity of fungal genera isolated from healthy leaves of aroeira. The phylogenetic tree generated by Maximum Likelihood of multi-gene sequences clustered LGMF651 and LGMF658 strains as *Diaporthe terebinthifolii* (FIGURE 14).

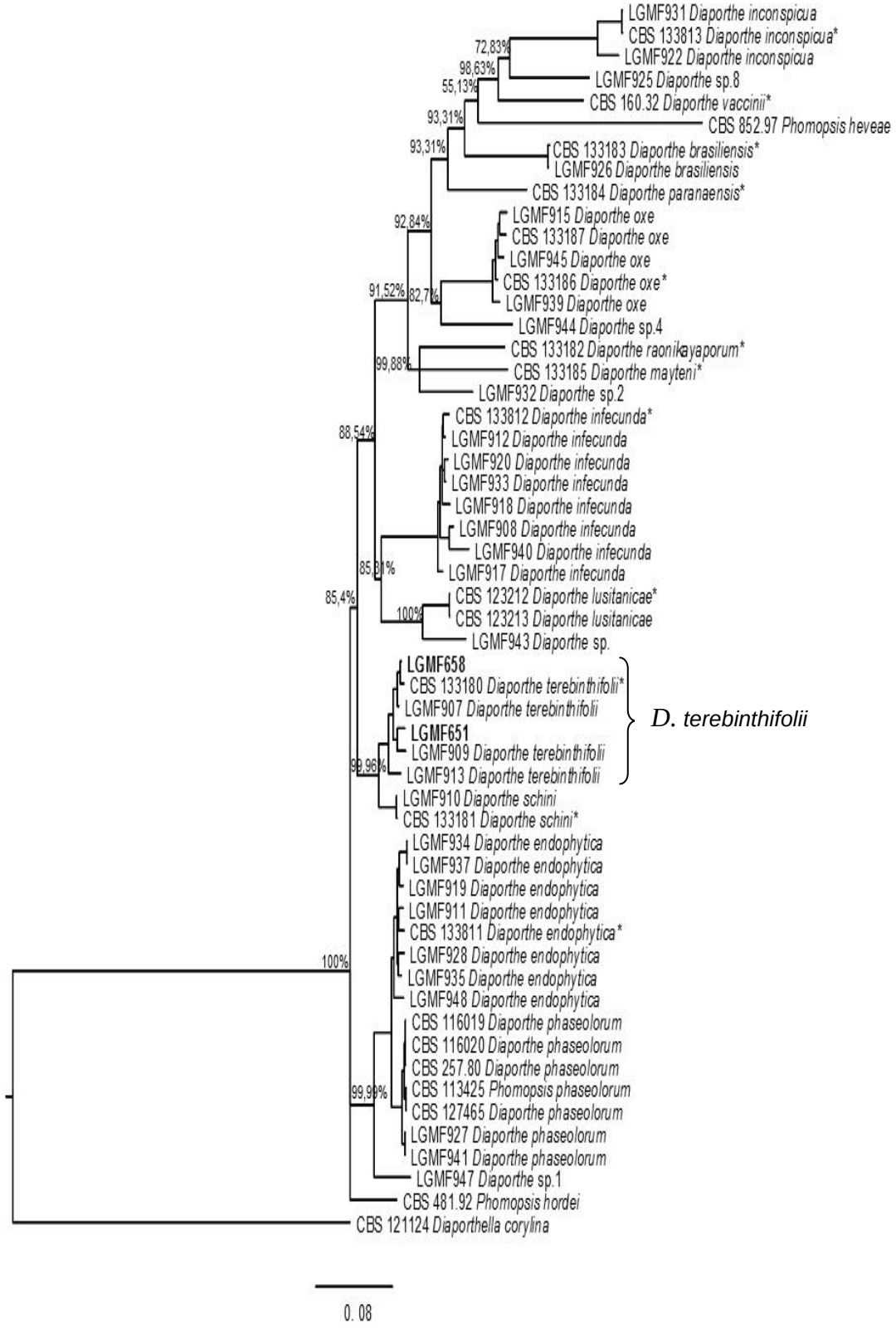

FIGURE 14. Bayesian analysis showing the position of the isolates LGMF651 and LGMF658 - printed in bold. The data matrix had 55 taxa and 2488 characters. Outgroup: *Diaporthella corylina* (CBS 121124).
*ex-type strain

5.4.2 FUNGI BIOLOGICAL ASSAYS

First of all, the isolates selected for the study were challenged directly against the phytopathogen. It was verify that inhibition of mycelial growth of *P. citricarpa* was more effective when there was the influence of compounds released into the medium, in pairing method (TABLE 4). Fact that subsequently directed the methodology for obtaining fungal metabolites, performed with solvent extraction from the culture media. The methodologies that verify the presence of bioactive volatile compounds, plates overlapping and plate with a division, also showed interesting results, but not such expressive as in the pairing (TABLE 4). Among the methods for study volatile metabolites action on the mycelial growth of the pathogen overlapping plates proved to be more responsive than the plate with the division (TABLE 4).

TABLE 4. Percentage inhibition of mycelial growth of *Phyllosticta citricarpa* induced by two endophytic fungi (*Diaporthe terebinthifolii*) through three confrontation methodologies*

D. terebinthifolii	Isolate LGMF658		Isolate LGMF651	
Bioassay	7 days	14 days	7 days	14 days
Pairing	NE	92,45	NE	71,87
Plates overlapping	71,15	75,34	57,69	60,27
Plate with a division	52,38	72,34	28,57	44,68

* All results are expressed in percent (%).
NE = not evaluated

5.4.3 BIOLOGICAL ASSAYS USING EXTRACTS

5.4.3.1 Mycelial growth inhibition

Inhibition of mycelial growth of *P. citricarpa* by extracts of the two strains tested was higher for the rice solid medium, suggesting a higher concentration of the compounds of interest (TABLE 5).

TABLE 5. Percentage of growth mycelial inhibition of *Phyllosticta citricarpa* by *Diaporthe terebinthifolii* extracts (10mg/mL) produced in different media*

MEDIA	Isolate LGMF658	Isolate LGMF651
Rice	72.50	71.19
Mueller-Hinton broth	63.10	70.00
Czapeck	57.76	20.00
Potato dextrose broth	48.39	0.00
MPE	30.95	2.38
Corn	6.48	46.51

*All values are expressed in percentage

5.4.3.2 Growth inhibition of the pathogen *in vivo* and broth microdilution (minimum inhibitory concentration - MIC)

Three portions were obtained from the extract produced in large scale (LGMF658 in 1600 g of rice medium): petroleum ether, dichloromethane and ethyl acetate. Only dichloromethane portion (10 mg/mL) retained the activity against *P. citricarpa*, reducing in 55,55 % the development of the pathogen on leaves; the other portions (petroleum ether and ethyl acetate) did not showed any inhibitory potential. So, the dichloromethane portion was subjected to column chromatography yielding ten fractions (fractions I-X).

The chromatographic fraction (VI) of the dichloromethane portion extended the protection on orange leaves at 100 %, in a reduced concentration of 0.5 mg/mL (FIGURE 15). The active fraction (VI) presented 0.003 µg/mL as minimum inhibitory concentration against the germination of *P. citricarpa* conidia, compared with 2.96 µg/mL by glifosinate. The others chromatographic fractions did not protected the leaves against the development of the pathogen. The portion of the fraction (VI) which maintained the activity it was the subfraction C (10-15 min), it presented on TLC three bands (C1, C2 and C3). However, the MIC of the subfraction C was 17.3 µg/mL, greater than the MIC for the fraction (VI), indicating a synergism among the mixture of compounds present in fraction (VI) (FIGURE 16).

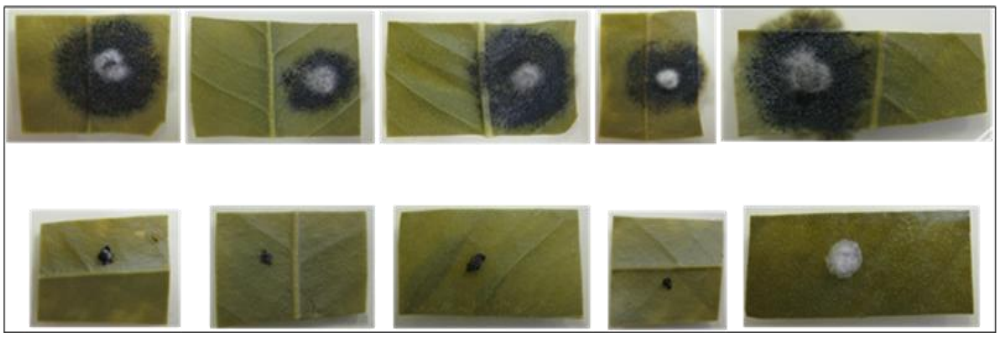

FIGURE 15. Growth inhibition of *Phyllosticta citricarpa in vivo* (leaves of orange tree). A = Negative control; B = chromatographic fraction (VI) (5 mg/mL) of *Diaporthe terebinthifolii* - LGMF658 extract

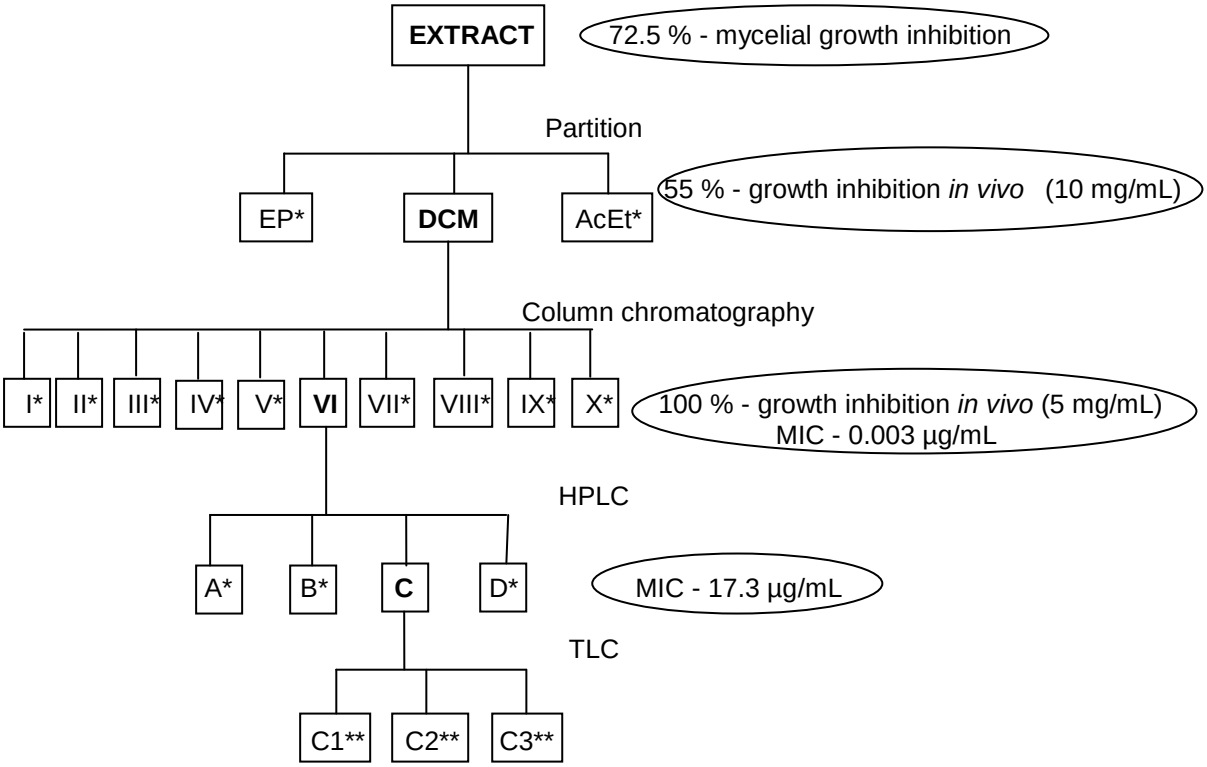

FIGURE 16. Diagram of the bioactivity guided purification of *Diaporthe terebinthifolii* - LGMF658 extract.
*Inactive fractions; in bold = active fractions
EP = petroleum ether; DCM = dichloromethane; AcEt = ethyl acetate

5.4.4 METABOLITES IDENTIFICATION

Among fraction's (VI) compounds of the *D. terebinthifolii* active extract against *P. citricarpa* it was identified by GC-MS: verbanol, verbanol acetate, hexadienol butanoate (2*E*-4*E*) and sesquicineol-2-one. The verbanol was also identified in the subfraction C demonstrating the importance of its presence in bioactivity. In the subfraction C other compound was also detected: the verbenyl acetate. The presence of verbanol was confirmed in the crude extract of the fungus, which also presented the following compounds: phenylethyl alcohol, methyl hexadecanoate and isoverbanol. In another active portion of crude extract against *Candida albicans* (data not shown) again it was detected by GC-MS the presence of verbanol, beside the compounds neoiso-verbanol, neoiso verbanol acetate and sesquicineol-2-one.

5.5 DISCUSSION

The *Diaporthe* (*Phomopsis*) endophytes are a rich source of bioactive secondary metabolites, including volatile and non-volatile. Some compounds produced by this genus have already had their phytopathogenic importance described. The production of volatile organic compounds (VOCs) produced by an endophytic *Phomopsis* sp. inhibited the growth of a diversity of plant pathogenic fungi. In the direct confront it significantly reduced the growth of *Sclerotinia sclerotiorum*, *Pythium ultimum*, *Aspergillus fumigatus*, *Rhizoctonia solani* and *Geotrichum candidum*. The artificial mixture of the identified VOCs was assayed against the phytopathogens and increased the action spectrum; the study did not evaluate the biological action against *P. citricarpa*. The main VOCs identified were sabinene and 1-butanol-3-methyl, also appeared benzeneethanol; 1-propanol-2-methyl and 2-propanone; many others compounds present in the mixture produced by the fungus were not identified (SINGH *et al.*, 2011).

A lactone, 8α-acetoxymultiplolide A, extracted from *Phomopsis* sp., an endophytic fungus, presented MIC values (µg/mL) of 31.25 against *Ophiostoma minus* and 62.5 against *Botrytis cinerea*, two plant pathogens (WU *et al.*, 2008). The species *P. longicolla* produces bioactive compounds against the bacteria *Xanthomonas oryzae* which causes a disease in rice (CHAESUNG *et al.*, 2010) and against the fungus *P. citricarpa* (FLORES *et al.*, 2013). The active compounds against *Xanthomonas oryzae* were identified as dicerandrol A (MIC 8 µg/mL), dicerandrol B (MIC 16 µg/mL), dicerandrol C (MIC >16 µg/mL), deacetylphomoxanthone B (MIC 4 µg/mL) and fusaristatin A (MIC 128 µg/mL). The study of the metabolites produced by *P. longicolla* against *P. citricarpa* suggests that the inhibition of mycelial growth is caused by 3-nitropropionic acid, the main constituent of the active fraction (FLORES *et al.*, 2013).

The identification of bioactive secondary metabolites produced by *D. terebinthifolii* against *P. citricarpa* is studied for the first time according to our knowledge. Abreu *et al*. (2012) suggest that the production of secondary metabolites in the genus *Phomopsis* may be species-specific, being, the metabolic profile, a possible new tool to delimit species. These data enhances the importance of the study of chemical constituents of the species. The production of secondary

metabolites by microorganisms depends on the growing conditions provided. So, different media were analyzed to obtain the most promising extract. The search approach of the best medium for the production of an extract does not discard the necessity of posterior optimization of cultive conditions to reach determinate product. But, while the bioactive composition is still unknown, it is an alternative to get an extract with greater concentration of the important metabolites or with less chemical interferents.

In tests of confrontation between endophyte and pathogen the LGMF658 isolate showed clear superiority in inhibiting the mycelial growth of *P. citricarpa*, although both fungi studied belong to the same specie. Furthermore, in the evaluating the activity of the extracts of both fungi it was observed that the strain LGMF651 suffered strong influence of the medium. So, the LGMF658 strain, less instable in active extract production and more potent in the direct confrontation against the pathogen, was choosed for large scale production.

From the identified secondary metabolites, the phenylethyl alcohol, the verbenyl acetate and the methyl hexadecanoate are highlighted by the known correlation with antimicrobial activity.

The phenylethyl alcohol is an antimicrobial compound that acts on DNA synthesis of fungi (LESTER, 1965), and breakdown the membrane permeability of bacteria (SILVER and WENDT, 1967). A mixture of VOCs produced by *Saccharomyces cerevisiae* containing phenylethyl alcohol had fungistatic effect on mycelial growth of *P. citricicarpa* and reduced the pathogen respiratory rate (FIALHO *et al.,* 2014). The compound also inhibited the mycelial growth of *Aspergillus niger, Penicillium notatum, Rhizopus nigricans* and *Neurospora crassa*; for *N. crassa* the inhibition of conidial germination was also evidenced (LESTER, 1965). Phenylethyl alcohol production by microorganisms was already reported for *Streptomyces* sp. (NARAYANA *et al.*, 2008), *Phoma* sp. (NAZNIN *et al.*, 2013), *A. niger* (ETSCHMANN *et al.*, 2014).

The verbenyl acetate, an oxygenated monoterpene, is a component of antimicrobial essential oils obtained from the aerial parts of *Artemisia kermanensis* (KAZEMI *et al.*, 2011) and *A. campestris* (GHORAB *et al.*, 2013). Comparing the fumigant activity of essential oils of the flowering aerial parts from *Artemisia giraldii* and *A. subdigitata*, the first, that has verbenyl acetate, was more active than the second, that do not present the compound (CHU *et al.*, 2012). The verbenyl acetate

is one of the main compounds of essential oil obtained from aerial parts of *Cotula cinerea*, which presents efficient antifungal activity against various *Candida* species (BOUZIDI *et al.*, 2011). These are evidences that verbenyl acetate can act as a bioactive or synergic compound. It were not found reports of biological activity by the isolated metabolite neither about the production of verbenyl acetate by microorganisms.

The methyl hexadecanoate (fatty acid - ester) has already been isolated as a secondary metabolite from an endophytic actinomycete of tomato plant, being considered one of the acaricides compounds from the endophyte extract (CHEN *et al.,* 2011). The compound was also detected in a mixture of volatile metabolites obtained from the endophytic bacterium *Burkholderia tropica,* which presented activity against phytopathogenic fungi (TENORIO-SALGADO *et al.,* 2013). One study suggests that the methyl hexadecanoate, with the synonymous of hexadecanoic acid methyl ester, is directly involved with the anti-inflammatory and antimicrobial activity of essential oils from *Perovskia abrotanoides* leaves (ASHRAF *et al.,* 2014). It is also part of the essential oil composition from *Myrica esculenta* stem bark, which has potent antimicrobial and significant anti-inflammatory activities (AGNIHOTRI *et al.*, 2012).

The results suggested a probable contribution of the verbanol with the antifungal activity of the extracts produced by the *D. terebinthifolii* (LGMF658) isolate. Verbanol is not commonly found as a natural product, the only relation found of its production by microorganisms was reported for *Aspergillus niger* (HUMPHREY and DEINDOERFER, 1962). No studies were found relating this alcohol with antimicrobial activity. We believe that the small number of studies concerning the compound is probably related to the fact that it is rare to find it as a natural product.

The MIC of the subfraction C was greater than the MIC for the fraction (VI), indicating a synergism among the mixture of compounds present in fraction (VI). The presence of other compounds in the fraction of interest suggests the possibility that other metabolites not identified could also be related to its main activity. However, according to the scientific evidence reported above, we can assert that if the compounds identified are not the mainly responsible for the activity of the product, they certainly help synergistically in the *P. citricarpa* inhibition. In synergism or in chemosensitization two or more active drugs with the same purpose produce a better

effect than the sum of the two separate over the common target (HARMAN *et al*., 2004; KIM *et al.*, 2013; MUSIOL *et al.*, 2014). In chemosensitization the activity of the agent may be so insignificant to the point of being ineffective when used alone, but when combined it can increase 10 times or more the antifungal potential of the another compound (CAMPBELL *et al.*, 2012). The use of this resource is interesting for making the fungus most vulnerable, reducing the concentration of antifungal that is necessary for an effective application against the pathogen and avoiding the induction of resistance (CAMPBELL *et al.*, 2012; KIM *et al.*, 2013).

An example of the synergic antifungal activity of VOCs was described by Ezra *et al.* (2004) who observed that none of the compounds of the endophytic *Muscodor albus* produced significant antifungal activity when used individually, but the VOCs combination was effective.

The complexity of the knowledge required understanding the interactions and the lack of appropriate methodologies for the study of synergism still makes this recourse be hardly applicable (MUSIOL *et al.*, 2014). As examples of chemosensitizing agents of agricultural antifungal with action scientifically proven *in vitro* we have: cinnamic aldehyde, octilgalato, 2,5-dihydroxybenzoic acid, berberine, 2,3-dihidroxibenzaldehyde, salicylaldehyde; but the action of these agents *in vivo* is still poorly studied (CAMPBELL *et al.*, 2012).

The investigation of compounds combinations produced by plants or microorganisms can only be achieved by analyzing the activity of crude and fractionated extracts, demonstrating the importance of works that seek for the induction of the total potential provided by the secondary metabolism of the specimen under study. The wealth present in the complex interaction of compounds that is naturally architected by living organisms is ignored when only the isolation of a specific active compound, without analyzing all the context of the property is considered.

The conidia are important inoculum sources of *P. citricarpa* in Brazil, playing a role in the increase and spread of CBS inside the contaminated tree or to its neighbors (SPÓSITO *et al.*, 2011). Knowing the importance of conidia in the disease cycle, it is suggested that the active fraction (VI) obtained in this study, with expressive biological activity on the inhibition of the germination of conidia, as well as, on the development of the disease on the orange leaves, may be considered an

alternative to help in the control of CBS. Therefore, it should be investigated in field and chemical composition studies.

In addition, taking into consideration that there is a need for control of CBS in apparently healthy fruit transported in containers to other continents because of the incubation period of the disease, the fact that the isolated compounds are volatile compounds makes it extremely promising for the development of a product for this purpose if its relationship with the inhibition of *P. citricarpa* be confirmed.

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6 CONSIDERAÇÕES FINAIS

Os fungos endofíticos isolados das folhas de *Schinus terebinthifolius* demonstraram potencial biotecnológico pela produção de extratos ativos tanto para área médica como agronômica.

A determinação das condições de cultivo demonstrou ser essencial para a obtenção dos metabólitos secundários de interesse.

Contra patógenos humanos, a partir de um isolado de *Alternaria* sp. Sect. Alternata, foram obtidas duas frações de extrato com atividade antimicrobiana, inclusive contra *Staphylococcus aureus* resistente a meticilina. Hexadecanoato de metila e um alcaloide pirrolopirazina foram identificados nas frações ativas do isolado de *Alternaria* sp. Sect. Alternata.

De um isolado de *Diaporthe terebinthifolii* foi extraída uma fração de extrato capaz de conter a proliferação de *Phyllosticta citricarpa* em folhas de laranjeira, e com uma concentração inibitória mínima de 0.003µg/mL sobre conídios do mesmo patógeno. Foram identificados alguns compostos de interesse produzidos pelo endófito: álcool fenetílico, verbanol, hexadecanoato de metila e acetato de verbenil.

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